THE UNIVERSITY *of* EDINBURGH

THE CARDIOVASCULAR EFFECTS OF APELIN

IN VIVO IN MAN

BY

Alan Gordon Japp

A thesis presented for the degree of Doctor of Philosophy at the

University of Edinburgh

2012

ABSTRACT

Background The apelin system is a novel peptidic pathway widely expressed in the heart and vasculature. In preclinical studies, apelin receptor agonism mediates nitric oxide-dependent vasodilatation, reduces ventricular preload and afterload and potently increases myocardial contractility. In preclinical models of heart failure, expression of the apelin pathway is down regulated but the haemodynamic effects of apelin receptor agonism are preserved. These changes in expression appear to be paralleled in patients with chronic heart failure but the cardiovascular actions of apelin *in vivo* in man are, to date, unknown. Detailed clinical investigation is therefore required to establish the role of apelin in human cardiovascular physiology and pathophysiology and to explore the therapeutic potential of apelin receptor agonism in patients with heart failure.

Objectives Through a series of *in vivo* clinical studies: 1) to establish the direct vascular actions of apelin in the peripheral venous, peripheral arterial and coronary arterial circulations; 2) to determine the contribution of the endothelium-derived vasodilators, nitric oxide and prostacyclin, to the vascular actions of apelin; 3) to establish the effects of apelin on cardiac contractility and systemic haemodynamics; 4) to compare the direct vascular and systemic haemodynamic effects of the full-length mature apelin peptide, apelin-36, with a shorter, biologically active carboxyl (C)-terminal fragment, (Pyr^{1})apelin-13); and 5) to establish whether the local vascular and systemic haemodynamic effects of apelin are altered in patients with chronic heart failure.

Methods The cardiovascular effects of apelin were assessed in 32 healthy volunteers, 6 patients undergoing elective diagnostic coronary angiography, 18 patients with stable New York Heart Association (NYHA) class II-III chronic heart failure and 18 age- and sex-matched healthy controls. Dorsal hand vein tone was assessed by the Aellig hand vein technique during local intravenous infusions (0.1-3 nmol/min) of apelin-36, (Pyr^{1})apelin-13, and sodium nitroprusside (SNP; 0.6 nmol/min). Forearm blood flow was measured by venous occlusion plethysmography during intrabrachial infusions of apelin-36 and (Pyr^{1})apelin-13 (0.01-30 nmol/min) and subsequently in the presence or absence of a 'nitric oxide clamp' (nitric oxide synthase inhibitor, L-N^{G}-monomethylarginine (L-NMMA; 8 μmol/min), co-infused with SNP (90-900 ng/min)), or a single oral dose of aspirin (600 mg) or matched placebo. Coronary blood flow was evaluated by quantitative coronary angiography (QCA) and Doppler flow wire, and left ventricular pressures measured by pressure wire before and after intracoronary injection of apelin-36 (20 and 200 nM), 0.9% saline and glyceryl trinitrate (GTN) (100 μg). Blood pressure, heart rate, cardiac output and peripheral vascular resistance were assessed by sphygmomanometry and thoracic electrical bioimpedance (TEB) during systemic intravenous infusion of apelin-36 and (Pyr^{1})apelin-13 (30-300 nmol/min). Forearm blood flow and systemic haemodynamic responses to (Pyr^{1})apelin-13 in patients with chronic heart failure were then compared with age- and sex-matched healthy controls.

Results Although SNP caused venodilatation ($P<0.0001$), apelin-36 and (Pyr^{1})apelin-13 had no effect on dorsal hand vein diameter ($P=0.2$). Both apelin isoforms caused vasodilatation in forearm resistance vessels ($P<0.0001$) but the offset was slower with apelin-36. (Pyr^{1})apelin-13-mediated vasodilatation was attenuated by the nitric oxide clamp ($P=0.004$) but unaffected by aspirin ($P=0.7$). Intracoronary bolus of apelin-36 increased coronary blood flow and the maximum rate of rise in left ventricular pressure, and reduced peak and end-diastolic left ventricular pressures (all $P<0.05$). Both (Pyr1)apelin-13 and apelin-36 increased heart rate and cardiac output whilst reducing peripheral vascular resistance ($P<0.01$ for all) with no overall effect on blood pressure. Intrabrachial infusions of (Pyr^{1})apelin-13, acetylcholine and SNP caused forearm vasodilatation in patients and controls ($P<0.0001$ for all). Vasodilatation to acetylcholine ($P=0.01$) but not apelin

(P=0.3) or SNP (p=0.9) was attenuated in patients with heart failure. Systemic infusions of (Pyr^1)apelin-13 increased cardiac index and lowered mean arterial pressure and peripheral vascular resistance index in patients and matched controls (all P<0.01) but increased heart rate only in controls (P<0.01).

Conclusions Although having no apparent effect on venous tone, acute apelin receptor agonism causes peripheral and coronary vasodilatation and increases cardiac contractility and output. Local vascular and systemic haemodynamic responses to apelin are preserved in patients with stable symptomatic chronic heart failure. The apelin system merits further clinical investigation to determine its role in cardiovascular homeostasis and represents a novel potential therapeutic target for patients with heart failure.

To Deepa and Dylan

CONTENTS

ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ANOVA	Analysis of variance
APJ	Apelin receptor
AT1	Angiotensin II type 1
AUC	Area under the curve
AVP	Arginine vasopressin
BNP	Brain natriuretic peptide
Ca^{2+}	Calcium
cAMP	cyclic adenosine monophosphate
CNS	Central nervous system
C-terminus	Carboxyl terminus
dP/dtmax	Maximum rate of rise in pressure
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERKs	Extracellular-regulated kinases
FBF	Forearm blood flow
GPCR	G protein-coupled receptor
GTN	Glyceryl trinitrate
IP3	1,4,5-trisphosphate
L-NAME	N^{ω}-nitro-L-arginine-methyl ester
L-NMMA	L-N^{G}-monomethyl-arginine
LVDT	Linear variable differential transformer
LVEDP	Left ventricular end-diastolic pressure
LV max	Maximum left ventricular systolic pressure

mRNA	Messenger ribonucleic acid
NCX	Na^+/Ca^{2+} exchanger
NHE	Na^+/H^+ exchanger
N-terminus	Amino terminus
HA	New York Heart Association
PIP2	Phosphatidylinositol 4,5-bisphosphate
PVN	Paraventricular nuclei
QCA	Quantitative coronary angiography
RAAS	Renin-angiotensin-aldosterone system
SEM	Standard error of the mean
SNP	Sodium nitroprusside
Sp1	Specificity protein 1
TEB	Thoracic electrical bioimpedence

DECLARATION

I hereby declare that the work described in this thesis was performed entirely by me with the following exceptions. The cardiac catheterisation procedures described in section 2.6 were carried out by Professor David Newby and Dr Nicholas Cruden. The apelin assays were performed by Professor Ian Megson, University of Highlands and Islands. All other assays were carried out by Neil Johnston, University of Edinburgh. Two of the thoracic bioimpedance studies described in Chapter 4 were performed by Dr Gareth Barnes. All of the work presented in this thesis has been published in peer-reviewed journals and no material has previously been submitted for any other professional degree or qualification.

Alan Gordon Japp

12th September 2012

ACKNOWLEDGEMENTS

I would like to thank Professor David Newby for the opportunity to conduct this research and for his guidance, encouragement and patience both during and after my years as a Research Fellow. I would also like to thank Dr Flapan for his support and supervision, and Dr Nicholas Cruden for his advice and practical support. In addition, I would like to acknowledge the helpful comments and suggestions provided by Professor Keith Fox, Dr Martin Denvir and Professor David Webb.

I am very grateful to the British Heart Foundation for supporting me both through their Scholarship Scheme and with a Clinical Research and Training Fellowship and to Chest Heart and Stroke Scotland for their support via a Project Grant.

I extend my warmest thanks to my research colleagues within the Centre for Cardiovascular Sciences and to staff at the Clinical Research Facilities of the Royal Infirmary of Edinburgh and Western General Hospital for their invaluable assistance and good humour.

I would like to thank Neil Johnston and Professor Iain Megson for performing the neurohormonal and apelin assays respectively and Jean Cunningham for her help with the formatting and production of this thesis.

Finally, I would like to thank my wife, Deepa, for her patience and encouragement and dedicate this thesis to her and our son, Dylan.

CHAPTER 1

INTRODUCTION:

THE APELIN SYSTEM IN HEALTH AND HEART FAILURE

Japp AG, Newby DE.
The apelin-APJ system in heart failure:
Pathophysiologic relevance and therapeutic potential.
Biochem Pharmacol 2008;**75**:1882-1892.

1.1 OVERVIEW OF THE APELIN SYSTEM

In 1993 a novel G protein-coupled receptor (GPCR) was identified through homology cloning by O'Dowd *et al* and named 'APJ' [O'Dowd *et al* 1993]. It remained an 'orphan' receptor until 1998 when its endogenous ligand was identified from bovine stomach extracts. The ligand, a 36 amino acid peptide, was named 'apelin' (APJ endogenous ligand) [Tatemoto *et al* 1998]. Subsequently, in 2008, the APJ receptor was renamed the 'apelin receptor' by the International Union of Pharmacology [Pitkin *et al* 2010a]. Since its discovery, the apelin system has emerged as an important regulator of cardiovascular homeostasis that may play a role in the pathophysiology of heart failure and represents an exciting target for the development of new therapies [Kleinz and Davenport 2005; Sorli *et al* 2006; Japp and Newby 2008].

1.2 THE APELIN RECEPTOR

1.2.1 STRUCTURE OF THE APELIN RECEPTOR

The apelin receptor is highly conserved across a range of species including human, rat, mouse, cow and rhesus macaque [Pitkin *et al* 2010a]. The human apelin receptor gene is located on the long arm of chromosome 11 and encodes a 380 amino acid GPCR with seven transmembrane-spanning domains [O'Dowd *et al* 1993]. It most closely resembles the angiotensin II type 1 (AT_1) receptor, with 54% sequence homology in the transmembrane regions, but does not bind angiotensin II. Radioligand binding studies in human tissue utilising [^{125I}]-(Pyr^{1})apelin-13 have

suggested a single receptor population with no evidence of distinct receptor subtypes [Katugampola *et al* 2001].

1.2.2 TRANSCRIPTIONAL REGULATION OF THE APELIN RECEPTOR

The transcriptional regulation of the apelin receptor gene appears to be complex and, at the time of writing, remains poorly understood. Physiological stimuli for apelin receptor synthesis include acute and chronic stress [O'Carroll *et al* 2003], salt loading [O'Carroll and Lolait 2003], water deprivation [Azizi *et al* 2008] and hypoxia [Sheikh *et al* 2008]. At the molecular level, a TATA-less promoter region within the gene has been identified [O'Carroll *et al* 2006]. The transcriptional factor**,** Specificity protein 1 (Sp1), which initiates transcription of several genes whose promoters lack a TATA box [Pugh *et al* 1991], also plays a major role in activation of the apelin receptor promoter. Other factors that contribute to promoter activity include CCAAT/enhancer binding protein, oestrogen and glucocorticoid protein complexes [O'Carroll *et al* 2006].

1.2.3 INTRACELLULAR SIGNALLING MECHANISMS

Apelin inhibits forskolin-stimulated production of cyclic adenosine monophosphate (cAMP) in Chinese hamster ovary cells, transfected with the apelin receptor, suggesting that the receptor couples to inhibitory G proteins (G_i) [Tatemoto *et al* 1998]. Apelin peptides also induce Ras-independent activation of extracellular-regulated kinases (ERKs) via protein kinase C [Masri *et al* 2002] as well as activation of p70S6 kinase (an important regulator of translation and cell cycle progression) through ERK- and Akt-dependent phosphorylation pathways

[Masri *et al* 2004]. These signalling cascades are inhibited by pertussis toxin implying that they too are mediated by coupling of the apelin receptor to G_i. On the other hand, the inotropic action of apelin is only partially suppressed by pertussis toxin and appears to involve activation of phospholipase C and protein kinase C [Szokodi *et al* 2002], a pathway characteristically activated by G_q proteins [Neves *et al* 2002]. This raises the possibility that the receptor may couple to G_q proteins in addition to G_i proteins. One well-established effect of phospholipase C activation is to increase intracellular production of inositol 1,4,5-trisphosphate (IP3) through hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) [Neves *et al* 2002]. This pathway may account for the observed increase in intracellular calcium (Ca^{2+}) concentrations in human NT2-N neurons on exposure to apelin [Choe *et al* 2000]. Activation by apelin peptides causes internalisation of the apelin receptor in a dose-dependent manner [Zhou *et al* 2003]. Although the receptor can mediate signal transduction from the cell surface, internalisation appears to be critical for activation of some downstream signalling pathways including those involved in the depressor response to apelin *in vivo* [El Messari *et al* 2004]. Receptor internalisation is also likely to underpin the desensitisation of several apelin-mediated signalling cascades observed following repeated ligand stimulation [Zhou *et al* 2003; Masri *et al* 2006]. Lee *et al* have previously demonstrated nuclear localisation of the apelin receptor [Lee *et al* 2004] suggesting the possibility that its effects may extend beyond the activation of intracellular cascades to transcriptional regulation, similar to other GPCRs such as AT_1 [Eggena *et al* 1993]. Finally, the AT_1 and apelin receptors have recently been shown to be capable of forming heterodimers [Chun *et al* 2008] with consequences for downstream signalling. The formation of heterodimers is promoted

by angiotensin II but unaffected by apelin and is the first apelin-independent action of the apelin receptor to be described. Such ligand-independent interactions provide one possible mechanism to account for observed phenotypic differences in apelin null and apelin receptor null animal models.

1.3 APELIN PEPTIDES

1.3.1 ENDOGENOUS APELIN

The human apelin gene, located on the long arm of the X chromosome, encodes a 77 amino acid prepropeptide that undergoes enzymatic cleavage to yield shorter active peptides (Figure 1.1) [Tatemoto *et al* 1998]. To date, the apelin receptor is the only receptor with which apelin peptides are known to bind and interact [Pitkin *et al* 2010a]. The full-length mature apelin peptide comprises 36 amino acids (apelin-36), but shorter fragments of its carboxyl (C)-terminus, consisting of 17 and 13 amino acids, have also been detected *in vivo* and exhibit biological activity [Tatemoto *et al* 1998; Habata *et al* 1999; Hosoya *et al* 2000]. Apelin-13 undergoes a pyroglutamate substitution at its amino (N)-terminus; a common post-translational modification that preserves biological activity by rendering the peptide more resistant to enzymatic degradation [Van Coillie *et al* 1998]. (Pyr^{1})apelin-13 is the most abundant isoform in cardiac tissue and plasma [Maguire *et al* 2009], whilst apelin-36 predominates in bovine colostrum [Hosoya *et al* 2000]. These two peptides also display different pharmacodynamic properties: (Pyr^{1})apelin-13 exhibits greater affinity for the apelin receptor but apelin-36 is less readily displaced [Hosoya *et al* 2000]; apelin receptors internalised into the cell following stimulation with

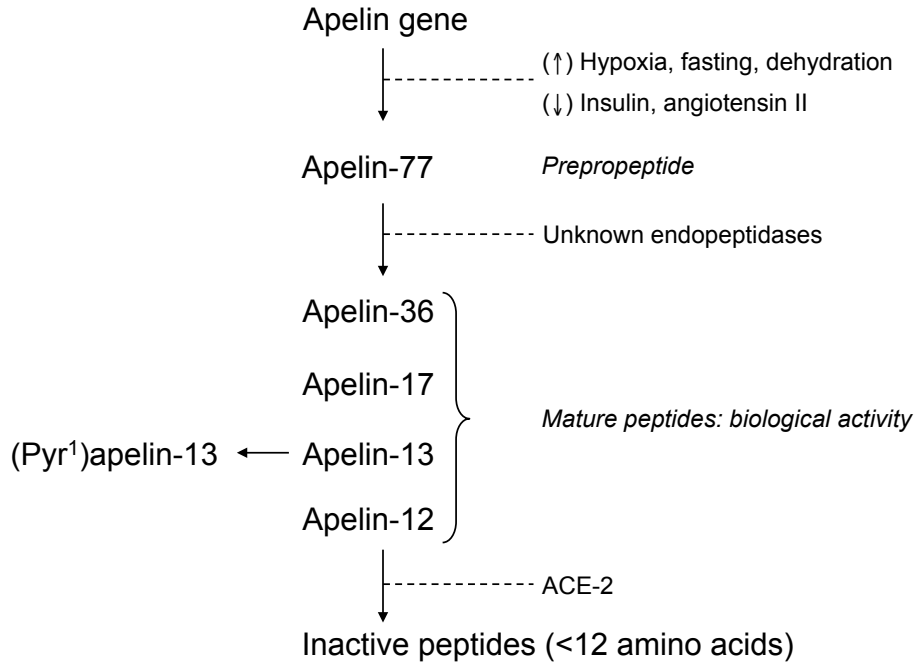

Figure 1.1 Apelin synthesis, post-translational processing and metabolism.
ACE - angiotensin-converting enzyme.

(Pyr[1])apelin-13 are rapidly recycled to the cell surface whereas those bound by apelin-36 remain within the cell cytoplasm for at least 2 hours [Zhou *et al* 2003]. In keeping with these findings, (Pyr[1])apelin-13 is the more potent isoform in competitive inhibition assays, but the biological effects of apelin-36 are more sustained [Hosoya *et al* 2000, Masri *et al* 2006]. The distinct physiological roles of the different apelin peptides remain uncertain.

Expression of the apelin gene in several tissues is increased in response to hypoxia under the regulation of hypoxia-inducible factor-1 [Ronkainen *et al* 2007; Sheikh *et al* 2008]. In breast tissue, there is up regulation of apelin synthesis during lactation that is mediated by upstream stimulatory factor-1 [Wang *et al* 2006], a fairly ubiquitous transcription factor involved primarily in energy metabolism and cellular proliferation [Corre and Galibert 2006]. In adipocytes, apelin gene expression is inhibited in the fasting state and stimulated by refeeding possibly through changes in the plasma concentrations of insulin and counter-regulatory hormones [Boucher *et al* 2005; Wei *et al* 2005]. Finally, in magnocellular neurons of the hypothalamus, apelin is up regulated by dehydration, through a mechanism that may involve arginine vasopressin (AVP) [Reaux-Le Goazigo *et al* 2004].

Less is known about the mechanisms regulating the post-translational processing of apelin including the proteolytic cleavage of the longer apelin isoforms to shorter C-terminal fragments and the pyroglutamine modification of apelin-13. One enzyme implicated in the processing of apelin peptides is angiotensin-converting enzyme (ACE) type 2, a carboxypeptidase that negatively regulates the

renin-angiotensin-aldosterone system (RAAS) by cleaving angiotensin II to the biologically inactive peptide angiotensin 1-9 or angiotensin 1-7 [Hamming *et al* 2007]. ACE type 2 has been reported to hydrolyse both apelin-13 and apelin-36 with high catalytic efficiency [Vickers *et al* 2002]. However, the physiological significance of this step remains unclear and recent *in vitro* data suggest it may not lead to inactivation of the peptide [Pitkin *et al* 2010a].

1.3.2 SYNTHETIC APELIN RECEPTOR AGONISTS

Several other fragments of the apelin peptide activate the apelin receptor and produce biological effects although their presence *in vivo* has yet to be established. The C-terminal portion of the apelin peptide is highly conserved across species and appears to be critical to biological activity: synthetic peptide fragments containing the final 12 residues of the C-terminus, including apelin-19, apelin-16 and apelin-12, display agonist activity whilst those shorter than 12 amino acids are biologically inert [El Messari *et al* 2004]. Recently, Iturrioz *et al* have developed the first non-peptidic apelin agonist, E339-3D6, an important step toward exploring the therapeutic potential of apelin analogues [Iturrioz *et al* 2010].

1.3.3 APELIN RECEPTOR ANTAGONISTS

Apelin-13(F13A), a synthetic analogue of apelin-13 with an alanine substitution at the C-terminus, was initially reported to antagonise the depressor effect of apelin-13 in rats *in vivo,* without affecting basal blood pressure [Lee *et al* 2005]. However other investigators have been unable to demonstrate its antagonistic activity *in vitro* [Fan *et al* 2003; Medhurst *et al* 2003]. In recent months, and subsequent to the

undertaking of this thesis, Macaluso *et al* have developed the first small-molecule apelin antagonist, cyclo(1-6)CRPRLC-KH-cyclo(9-14)CRPRLC that competitively inhibits apelin-induced receptor internalisation and inhibition of cAMP production in cell lines transfected with the human apelin receptor [Macaluso *et al* 2011].

1.4 LOCALISATION OF THE APELIN SYSTEM

1.4.1 APELIN RECEPTOR LOCALISATION

In both rat and human, the apelin receptor is widely represented in the central nervous system (CNS) and a variety of peripheral tissues [Pitkin *et al* 2010a]. Within the rat brain, expression is particularly high in magnocellular neurons of the supraoptic and paraventricular hyopthalamic nuclei, where it colocalises with AVP [De Mota *et al* 2004; Reaux-Le Goazigo *et al* 2004]. Within some peripheral tissues, such as lung, kidney and adrenal gland, expression appears to be restricted to the vasculature [Kleinz *et al* 2005], though apelin receptors have also been detected in enterochromaffin-like gastric cells [Lambrecht *et al* 2006], pancreatic islet cells [Sörhede Winzell *et al* 2005], hepatic stellate cells [Melgar-Lesmes *et al* 2011], adipocytes [Wei *et al* 2005], osteoblasts [Xie *et al* 2006] and T-lymphocytes [Choe *et al* 1998]. Within the human vasculature, apelin receptor protein is detectable in large conduit arteries (radial, mammary, coronary) and veins (saphenous), and in small vessels from a variety of vascular beds [Kleinz *et al* 2005]. Receptors are located predominantly within the endothelium but are also present, at lower levels, in vascular smooth muscle cells. Within the human heart, apelin receptors are present in

endocardial endothelial cells and, at lower levels, in cardiomyocytes [Kleinz *et al* 2005].

1.4.2 APELIN PEPTIDE LOCALISATION

The expression pattern of apelin is closely related to that of the apelin receptor. Within the human vasculature, apelin is detectable in large conduit vessels including radial artery, left internal mammary artery, coronary artery and saphenous vein, as well as renal, adrenal and pulmonary vessels [Kleinz and Davenport 2004]. However, unlike the receptor, its expression appears to be restricted to the endothelium [Kleinz and Davenport 2004; Sheikh *et al* 2008]. Recently, apelin protein has been detected within atherosclerotic plaque of human coronary artery where it colocalises with both the apelin receptor and with markers of smooth muscle cells and macrophages [Pitkin *et al* 2010b]. Within the human heart, expression appears to be restricted to endocardial endothelial cells [Kleinz and Davenport 2004].

1.4.3 AUTOCRINE, PARACRINE OR ENDOCRINE SYSTEM?

The colocalisation of apelin and the apelin receptor in many tissues suggests a possible autocrine or paracrine signalling pathway. In keeping with this, the receptor and ligand exhibit co-ordinated tissue-specific changes in expression under different physiological conditions such as hypoxia [Sheikh *et al* 2008] and obesity [Dray *et al* 2010]. Accurate quantification of apelin concentration in plasma has been confounded by limitations in the sensitivity and use of commercially available assays [Japp and Newby 2008]. This has led to marked discrepancies in measured concentrations with around 40-fold variation (90-3,580 pg/mL) being reported

among healthy control populations [Földes *et al* 2003; Chong *et al* 2006]. However this is likely to reflect insufficient extraction of plasma proteins in some studies, resulting in non-specific binding and reporting of falsely elevated plasma apelin concentrations [Barnes *et al* 2010]. Recent studies combining improved assay technology with more exact methodology suggest concentrations in the subpicomolar range, lower than would be expected for a circulating peptide [Pitkin *et al* 2010b]. The main source of plasma apelin is currently unclear although adipose tissue and the vascular endothelium are likely to contribute.

1.5 PHYSIOLOGICAL ROLES OF THE APELIN SYSTEM

1.5.1 OVERVIEW OF PHYSIOLOGICAL FUNCTIONS

In keeping with its widespread pattern of expression, the apelin system has been implicated in a variety of physiological functions. Whilst the clearest evidence to date for a physiological role is in the regulation of cardiovascular homeostasis, apelin signalling appears to influence a number of other physiological processes [Sorli *et al* 2006; Barnes *et al* 2010; Pitkin *et al* 2010a]. In addition to the specific areas discussed below, emerging evidence suggests a role for the apelin pathway in thermoregulation [Jászberényi *et al* 2004], immune function [Cayabyab *et al* 2000], bone metabolism [Xie *et al* 2007], gut hormone secretion [Lambrecht *et al* 2006] and the neuroendocrine stress response [O'Carroll *et al* 2003].

1.5.2 FLUID HOMEOSTASIS

In rats, the apelin system is highly expressed in regions of the CNS critical to osmotic regulation, including magnocellular neurons of the hypothalamic supraoptic and paraventricular nuclei (PVN) [O'Carroll and Lolait 2003; De Mota *et al* 2004]. Here, both apelin and its receptor colocalise with AVP, a key modulator of water homeostasis released during increased plasma osmolality or reduced plasma volume to promote water retention by renal collecting duct cells. Apelin synthesis is increased in response to water deprivation under the actions of AVP [O'Carroll *et al* 2003; Reaux-Le Goazigo *et al* 2004]. In turn, intracerebral injection of apelin directly inhibits AVP neuron activity and induces a diuresis with little or no apparent increase in sodium excretion [De Mota *et al* 2004]. In addition to its central interactions with AVP, apelin also exerts vasoactive effects on the renal microvasculature and may modulate fluid balance through direct effects on glomerular haemodynamics [Hus-Citharel *et al* 2008]. Recent studies in apelin receptor knockout mice appear to confirm an important physiological role for endogenous apelin signalling in fluid homeostasis: mice lacking the apelin receptor gene exhibit reduced drinking behaviour under basal conditions and an inability to concentrate urine in response to osmotic challenge [Roberts *et al* 2009]. To date, clinical data are limited but, in healthy volunteers, water loading results in elevated plasma apelin concentrations, whilst increased plasma osmolality causes plasma apelin concentrations to fall; in each case there is a reciprocal change in AVP concentration [Azizi *et al* 2008]. Taken together, these data suggest that apelin may play an important counter-regulatory role to AVP in fluid homeostasis.

1.5.3 ENERGY METABOLISM

Apelin is produced by adipose tissue and influences glucose metabolism [Boucher *et al* 2005]. In preclinical models, apelin inhibits insulin secretion by pancreatic islet cells [Sörhede Winzell *et al* 2005] and increases peripheral glucose uptake through a direct action on skeletal muscle [Yue *et al* 2010]. Accordingly, exogenous apelin in rodents reduces peak plasma glucose concentrations following a glucose load in both healthy and insulin-resistant animals [Dray *et al* 2008]. Furthermore, mice with deletion of the apelin gene exhibit impaired insulin sensitivity that is reversed by exogenous apelin supplementation [Yue *et al* 2010]. The effect of exogenous apelin on glucose handling in man is currently unknown. However, increases in plasma apelin concentrations are seen during oral glucose tolerance tests in healthy individuals, and those with insulin resistance or type 2 diabetes mellitus [Li *et al* 2006]. Apelin signalling also appears to regulate lipid metabolism. Apelin inhibits lipolysis in cultured adipocytes [Yue *et al* 2011] whilst mice with deletion of the apelin gene display increased plasma levels of free fatty acids and glycerol, an effect reversed by exogenous apelin replacement [Yue *et al* 2010]. Finally, in murine models, both endogenous and exogenous apelin appears to decrease adiposity without affecting food intake [Higuchi *et al* 2007; Yue *et al* 2010].

1.5.4 CARDIOVASCULAR DEVELOPMENT AND ANGIOGENESIS

In a variety of *in vitro* and preclinical *in vivo* models apelin exerts potent angiogenic and mitogenic effects [Kasai *et al* 2004; Cox *et al* 2006; Sorli *et al* 2007]. In several species, during embryonic development, apelin receptors are expressed in endothelial progenitor cells and rudimentary cardiovascular structures [Cox *et al* 2006; Zeng *et*

al 2007; Kidoya *et al* 2008] and endogenous apelin signalling appears to be essential for normal cardiovascular development. In the developing zebrafish [Scott *et al* 2007; Zeng *et al* 2007] and frog [Cox *et al* 2006], altered expression of either the ligand or receptor gene disrupts vascular development and produces morphological cardiac abnormalities. In mice deletion of the apelin gene during embryogenesis retards retinal vascular development [Kasai *et al* 2008], whilst knockout of the apelin receptor gene results in developmental cardiac defects [Charo *et al* 2009].

The angiogenic effects of apelin signalling appear to extend beyond foetal life. Both apelin and the apelin receptor are up regulated by hypoxia in a range of tissues [Ronkainen *et al* 2007; Sheikh *et al* 2008; Geiger *et al* 2011; Melgar-Lesmes *et al* 2011] and apelin signalling promotes neovascularisation of adipose tissue [Kunduzova *et al* 2008] and solid tumours [Sorli *et al* 2007]. Apelin deficiency has been shown to decrease the hypoxia-induced regeneration of vessels in zebrafish, [Eyries *et al* 2008] and to worsen necrosis in a model of hind limb ischaemia in mice [Kidoya *et al* 2010]. Furthermore, in the latter model, exogenous apelin administration increased angiogenesis and decreased necrosis following hind limb ischaemia.

1.6 VASCULAR EFFECTS OF APELIN

1.6.1 VASCULAR EFFECTS *IN VITRO*

Apelin activates endothelial nitric oxide synthase (eNOS) in cultured human umbilical vein endothelial cells [Ishida *et al* 2004] possibly via Akt phosphorylation, and stimulates nitric oxide production in rat aorta [Jia *et al* 2007]. In keeping with this, apelin causes vasorelaxation in *ex vivo* human mesenteric arteries that is attenuated by nitric oxide synthase but not cyclooxygenase inhibition [Salcedo *et al* 2007]. However, it also stimulates myosin-light chain phosphorylation in rat and mouse vascular smooth muscle [Hashimoto *et al* 2006], a key step in contraction and causes constriction *ex vivo* in human saphenous vein and internal mammary artery denuded of endothelium [Katugampola *et al* 2001]. Collectively these data suggest that apelin can act directly on receptors within vascular smooth muscle to induce contraction but that in the presence of functioning endothelium, this effect is outweighed by stimulation of local nitric oxide production via endothelial apelin receptors (Figure 1.2).

1.6.2 VASCULAR EFFECTS *IN VIVO* IN ANIMAL MODELS

Apelin was originally shown to cause a rapid and transient fall in mean arterial pressure following bolus injection in rats [Lee *et al* 2000]. This finding in rodents has been widely replicated [Reaux *et al* 2001; Tatemoto *et al* 2001; El Messari *et al* 2004; Ishida *et al* 2004; Lee *et al* 2005; Jia *et al* 2006] but is absent in mice with targeted knockout of the apelin receptor gene, confirming that it is mediated via

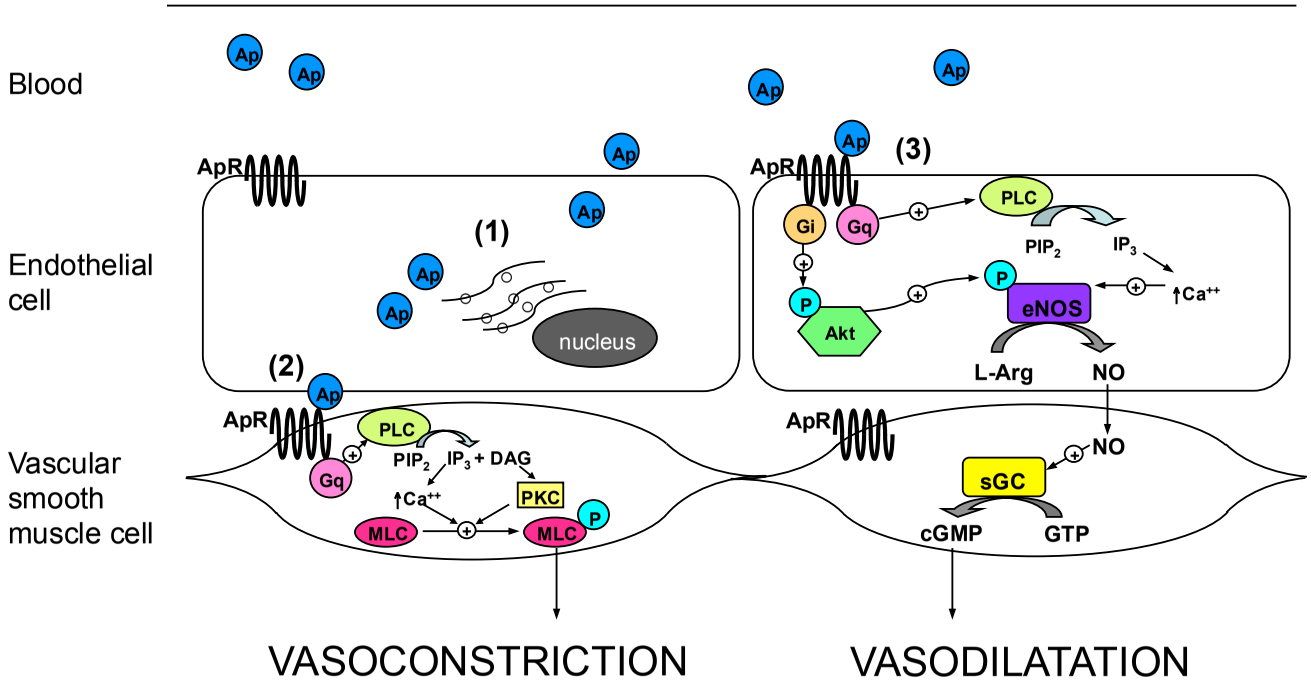

Figure 1.2 Proposed model of vascular effects of apelin system. **1)** Apelin is synthesised locally in endothelial cells then transported to luminal and basolateral cell membranes. **2)** Apelin peptides directly activate apelin receptors on vascular smooth muscle cells leading to vasoconstriction, possibly through coupling to a Gq protein. **3)** Locally released and circulating apelin molecules activate endothelial apelin receptors generating NO release; NO diffuses into smooth muscle cells producing vasodilatation. In the presence of intact endothelium the net effect is vasodilatation.
Ap - apelin; P - phosphate; Gi - inhibitory G protein; Gq - Gq protein; PLC - phospholipase C; PKC - protein kinase C; PIP2 - phosphatidylinositol 4,5-bisphosphate; IP3 - inositol 3,4,5 trisphosphate; DAG - diacylglycerol; NO - nitric oxide; sGC - soluble guanylate cyclase; eNos - endothelial nitric oxidie synthase; Ca^{++}- calcium; L-Arg - L-arginine; MLC - myosin light chain; cGMP - cyclic guanine monophosphate; GTP -guanine triphosphate.

the apelin receptor [Ishida *et al* 2004]. In keeping with *in vitro* data, the depressor response is also abolished by co-administration of N^{ω}-nitro-L-arginine-methyl ester (L-NAME), a nitric oxide synthase inhibitor, suggesting it occurs through nitric oxide-mediated arterial vasodilatation [Tatemoto *et al* 2001].

Bolus intravenous apelin injection reduces mean circulatory filling pressure [Cheng *et al* 2003], an accurate reflection of systemic venous tone [Pang 2000] implying that apelin can function as both an arterial and venous dilator. Indeed, by this measure, apelin is a more efficacious venodilator than either nitrates or hydralazine. These changes in vascular tone are matched by corresponding alterations in ventricular loading conditions: acute apelin administration reduces left ventricular end-diastolic area and left ventricular end-systolic pressure [Ashley *et al* 2005] To date there have been no reports of apelin-mediated vasoconstriction *in vivo*.

Currently there are no reports of the *in vivo* effects of apelin in man. However the only *in vivo* study in large mammals to date has raised the possibility that cardiovascular responses to apelin may exhibit interspecies differences. Acute bolus administration of apelin in an ovine model at equivalent doses to rodent studies elicited a biphasic haemodynamic response with an initial transient reduction in arterial pressure and rise in heart rate followed rapidly by an increase in blood pressure and concomitant fall in heart rate [Charles *et al* 2006]. Cardiac output was not measured early in this study but fell during the hypertensive phase, paralleling the reduction in heart rate and coinciding with rises in peripheral vascular resistance and right atrial pressure.

1.6.3 VASCULAR EFFECTS OF ENDOGENOUS APELIN

The lack of a suitable apelin receptor antagonist has prevented detailed characterisation of the role of endogenous apelin in the regulation of vascular tone. Murine models with targeted deletion of either the apelin [Kuba *et al* 2007] or apelin receptor [Ishida *et al* 2004] gene have normal basal blood pressure. However mice with apelin receptor deletion exhibit an exaggerated pressor response to angiotensin II [Ishida *et al* 2004]. Furthermore additional deletion of the apelin receptor in mice already lacking the AT_1 receptor causes an increase in basal blood pressure. Together, these effects suggest a possible contribution from endogenous apelin signalling to basal vascular dilator tone.

1.7 CARDIAC EFFECTS OF APELIN

1.7.1 CARDIAC EFFECTS OF APELIN *IN VITRO*

In keeping with the localisation of apelin receptors within the heart, apelin exhibits direct myocardial effects. In isolated rat hearts, apelin increases contractility at subnanomolar concentrations and augments the preload-induced increase in the maximum rate of rise in pressure (dP/dtmax) within the left ventricle [Szokodi *et al* 2002]. Apelin also induces sacromere shortening in individual cardiomyocytes obtained from both normal and failing myocardium [Farkasfalvi *et al* 2007] and increases contractility in isolated right ventricular trabeculae from failing rat hearts [Dai *et al* 2006]. Although these studies all support a positive inotropic role for apelin, there are discrepancies in the reported findings. Whilst the reported EC_{50}

value for apelin in normal intact rat hearts was 33 pM, making apelin the most potent inotrope yet studied, concentrations more than 1000-fold higher failed to elicit an increase in contractility in normal rat trabeculae, and produced only a very modest response in failing trabeculae. In addition the slow-onset sustained increase in contractility over a period of 30 minutes observed in the intact rat heart model, contrasts sharply with the transient response of only 1-2 minutes seen in single cardiomyocytes. Such discrepancies may be attributable to the different methodologies employed or regional variation in the density of apelin receptors within the heart. In isolated cardiomyocytes, the absence of mechanical load may have contributed to a more limited response, especially as apelin only increased preload-induced dP/dtmax in intact hearts, at higher loading conditions. Given the preferential localisation of both receptor and ligand in endocardial cells over cardiomyocytes, it is also tempting to speculate that signal transduction from activated endocardial apelin receptors might play a predominant role in mediating the inotropic effects of apelin. If so, preservation of this signalling pathway in intact hearts might account for the greater responses to apelin seen in this model. At the time this thesis was undertaken, there were no reports of the direct effects of apelin on human cardiac tissue. However Maguire *et al* [Maguire *et al* 2009] have recently confirmed potent inotropic activity *ex vivo* in human paced atrial strips.

1.7.2 CARDIAC EFFECTS OF APELIN *IN VIVO* IN ANIMAL MODELS

The inotropic effects of the apelin system in rodents extend to the *in vivo* setting where acute apelin infusion increases left ventricular dP/dtmax and cardiac output [Berry *et al* 2004; Jia *et al* 2006; Atluri *et al* 2007] as well as load-independent

measures of myocardial contractility such as ventricular elastance and preload recruitable stroke work [Ashley *et al* 2005]. Importantly, chronic administration also leads to an increase in cardiac output without inducing left ventricular hypertrophy [Ashley *et al* 2005].

1.7.3 THE ROLE OF ENDOGENOUS APELIN SIGNALLING IN CARDIAC REGULATION

Mice with targeted knockout of either the apelin or apelin receptor gene exhibit normal cardiac development and baseline haemodynamic parameters but have striking impairment of sarcomeric function accompanied by reduced cardiac contractility and diminished exercise capacity [Charo *et al* 2009]. Apelin null mice subjected to chronic pressure overload with aortic banding develop severe heart failure compared with wild-type mice and, even in the absence of aortic banding, develop progressive left ventricular dysfunction and dilatation from around 6 months of age [Kuba *et al* 2007]. Exogenous replacement of apelin in adult life prevents this age-related decline in cardiac performance, suggesting it is attributable to contemporary loss of apelin activity and not the manifestation of a latent developmental defect. In comparison to mice with deletion of the apelin gene, those with deletion of the apelin receptor exhibit poorer effort tolerance, earlier echocardiographic evidence of left ventricular dysfunction [Charo *et al* 2009] and reduced survival during embryogenesis [Charo *et al* 2009]. Such phenotypic differences suggest either ligand-independent effects of the apelin receptor or an undiscovered ligand. Taken together the above genetic data imply an important role for endogenous apelin signalling in maintaining cardiac performance both under basal conditions and particularly during conditions of cardiovascular stress.

1.7.4 MECHANISMS OF APELIN-MEDIATED INOTROPY

The mechanisms by which apelin exerts its inotropic effects have been only partially elucidated and remain the subject of debate. The effects are independent of angiotensin II, endothelin-1, catecholamines and nitric oxide release [Szokodi *et al* 2002] but appear to involve activation of the sacrolemmal Na^+/H^+ exchanger (NHE), probably through phospholipase C and protein kinase C-dependent pathways (Figure 1.3) [Szokodi *et al* 2002; Farkasfalvi *et al* 2007]. In single cardiomyocytes, NHE activity increases following exposure to apelin while, in intact rat hearts, the inotropic response to apelin is markedly attenuated by a specific inhibitor of NHE. Stimulation of NHE can lead to intracellular alkalinisation and sensitisation of cardiac myofilaments to intracellular Ca^{2+} [Karmazyn *et al* 1999]. In keeping with this, activation of NHE by apelin is accompanied by an increase in intracellular pH [Farkasfalvi *et al* 2007], whilst cytoplasmic Ca^{2+} transients are unchanged and perforated patch clamp recordings show that apelin does not alter voltage-gated Ca^{2+} channels in cardiomyocytes. Furthermore, in cardiomyocytes isolated from mice lacking either the apelin or apelin receptor gene, sarcomeric function is impaired without any corresponding alterations in intracellular calcium transients [Charo *et al* 2009]. These data suggest that apelin increases myocardial contractility by enhancing the sensitivity of myofilaments to activator Ca^{2+} rather than increasing intracellular Ca^{2+} concentrations. However, activation of NHE can also indirectly increase intracellular Ca^{2+} as the resulting accumulation of Na^+ within cells stimulates the reverse mode Na^+/Ca^{2+} exchanger (NCX) [Kentish 1999]. In intact rat hearts, inhibition of NCX also suppresses the apelin-induced inotropic response indicating

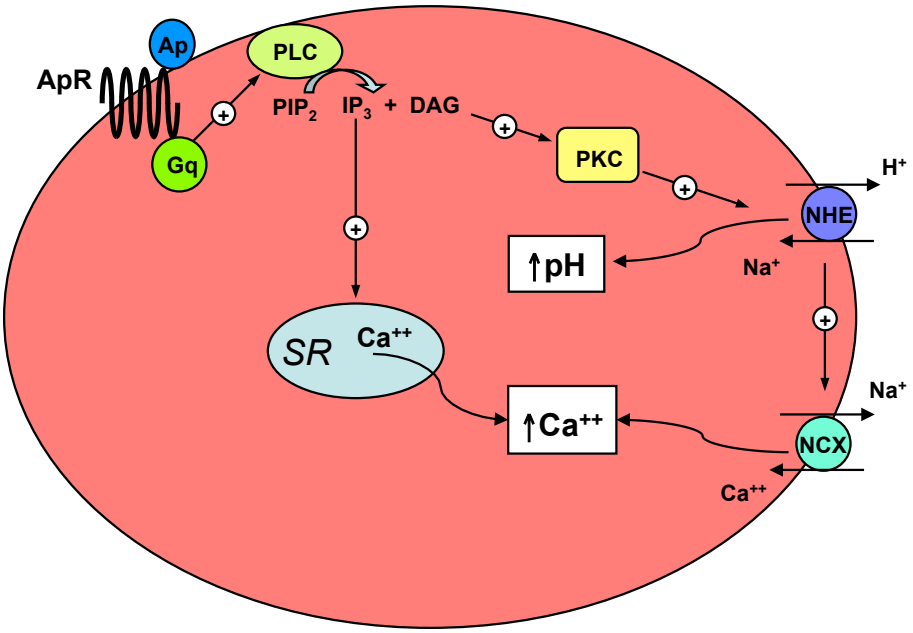

Figure 1.3 Possible intracellular mechanisms of apelin-mediated positive inotropic effects. Ap - apelin; ApR - apelin receptor; Gq - Gq protein; PLC - Phospholipase C; SR - sacroplasmic reticulum; Ca^{++} - calcium; NHE - Na^{+}/H^{+} exchanger; NCX - reverse $Na^{+/}Ca^{2+}$ exchanger; PIP2 - phosphatidylinositol 4,5-bisphosphate; IP3 - inositol 3,4,5 trisphosphate; DAG - diacylglycerol.

that this mechanism may contribute to apelin-mediated inotropic activity. Furthermore, in failing rat trabeculae, apelin failed to alter steady-state force-[Ca^{2+}]i relations but increased the amplitude of the intracellular Ca^{2+} transient [Dai *et al* 2006]. Thus the inotropic effects of apelin may involve increased intracellular Ca^{2+} availability in addition to enhanced myofilament responsiveness to Ca^{2+}.

1.8 THE APELIN SYSTEM IN HEART FAILURE

1.8.1 ALTERED EXPRESSION OF THE APELIN SYSTEM IN PRECLINICAL MODELS OF HEART FAILURE

Expression of apelin and its receptor is increased or maintained in animals with left ventricular hypertrophy and compensated heart failure but down regulated in those with severe, decompensated heart failure [Iwanaga *et al* 2006; Jia *et al* 2006]. This down regulation may be related to increased activity of the RAAS. In one heart failure model, several pharmacological treatments retarded the progression to chronic heart failure, but only AT_1 receptor antagonism prevented the down regulation of apelin receptor and ligand expression [Iwanaga *et al* 2006]. Furthermore, infusion of angiotensin II over 24 hours, even at subpressor doses, reduced both apelin and apelin receptor expression, and this effect was also abolished by concurrent AT_1 receptor blockade. Cardiac dilatation in advanced heart failure may also contribute to down regulation of the apelin system since cardiomyocytes subjected to mechanical stretch *in vitro* exhibit markedly reduced expression of both the apelin receptor and ligand [Szokodi *et al* 2002].

Regulation of the apelin pathway is altered by acute ischaemic injury. Apelin gene expression and secretion in isolated cardiomyocytes is increased by acute hypoxia through the hypoxia inducible factor pathway [Ronkainen *et al* 2007]. Accordingly, apelin expression is up regulated *in vivo* within 24 hours of myocardial infarction [Ronkainen *et al* 2007]. Endogenous cardiac apelin and the apelin receptor are increased in rats with ischaemic heart failure 6 weeks post myocardial infarction [Atluri *et al* 2007]. It is not clear whether the stimulus for this up regulation is ischaemia or the early onset of heart failure. In contrast, both receptor and ligand expression fall in a further rodent model of ischaemic myocardial injury caused by repeated isoprotenerol administration [Jia *et al* 2006]. This model produced extensive myocardial injury and very severe heart failure associated with hypotension and grossly elevated left ventricular end-diastolic pressure (LVEDP). Interestingly, while cardiac apelin receptor messenger ribonucleic acid (mRNA) levels were markedly down regulated in these rats, both tissue levels and overall apelin-binding capacity of the receptor within the heart were increased. This might reflect more efficient post-transcriptional processing of apelin receptor and / or diminished breakdown of existing receptors.

1.8.2 ALTERED EXPRESSION OF THE APELIN SYSTEM IN PATIENTS WITH HEART FAILURE

In keeping with the findings from preclinical models, expression of the apelin pathway is altered in patients with chronic heart failure. Initial reports suggested that plasma apelin concentrations were mildly elevated in the early stages of heart failure but fell with more advanced disease [Chen *et al* 2003; Földes *et al* 2003]. In support

of this, there are now several further reports of depressed plasma apelin concentrations in patients with advanced chronic heart failure [Chong *et al* 2006; Goetze *et al* 2006; Francia *et al* 2007]. Furthermore, in patients with severe chronic heart failure, the improvement in New York Heart Association (NYHA) symptom class and left ventricular ejection fraction seen following cardiac resynchronisation therapy is paralleled by restoration of normal plasma apelin concentration [Francia *et al* 2007]. Some subsequent studies have produced conflicting findings and, as described above, this may in part reflect significant limitations in currently available commercial assays for apelin. However careful scrutiny suggests that apparent inconsistencies may also be attributable to differences in study populations (Table 1.1). For example, Chong and co-workers reported that plasma apelin concentrations were lower in patients with chronic heart failure than in normal controls irrespective of NYHA symptom class or left ventricular ejection fraction [Chong *et al* 2006]. However these investigators studied a cohort of patients with disproportionately severe heart failure: 73% of patients were NYHA class III or IV and only 3% were NYHA class I. Moreover mean ejection fraction was 15% and, even in NYHA class II patients, it was a mere 18%. In contrast two subsequent studies have since claimed that plasma apelin concentrations are unaltered in patients with chronic heart failure compared with age-matched controls [Codognotto *et al* 2007; Miettinen *et al* 2007]. However in both of these studies, more than 95% of patients were in NYHA functional class I or II, and the average left ventricular ejection fraction was 40% and 42% respectively. Therefore, taken together, current data suggest that apelin expression is at least maintained and possibly augmented in mild, compensated heart failure but declines with advancing disease.

TABLE 1.1 Plasma concentration of apelin in patients with chronic heart failure

Authors	NYHA Class	Mean LVEF	Aetiology	Plasma apelin concentration
Chong *et al* 2006	III/IV (73 %) I/II (27%)	15.6%	Ischaemic and DCM	Decreased
Goetze *et al* 2006	Not stated	20%	Ischaemic and DCM	Decreased
Francia *et al* 2007	III/IV (100%)	25%	Ischaemic and DCM	Decreased
Foldes *et al* 2003	III (100%)	Not stated	Ischaemic	Decreased
Chen *et al* 2003	III/IV (51%) I/II (49%)	>25% (50%) <25% (50%)	Ischaemic and DCM	Increased in early stages; lower in severe disease
Miettinen *et al* 2007	IV (0%) III (3%) II (48%) I (49%)	40%	DCM	Normal
Codognotto *et al* 2007	I (100%)	42% (median)	DCM	Normal

NYHA - New York Heart Association; LVEF - left ventricular ejection fraction; DCM - dilated cardiomyopathy.

Apelin receptor mRNA levels within the left ventricle are reduced in patients with advanced heart failure due to idiopathic dilated cardiomyopathy but unaltered in patients with ischaemic cardiomyopathy [Földes *et al* 2003], perhaps reflecting the stimulatory effect of local hypoxia on receptor expression. On the other hand, apelin receptor density within the left ventricle is reduced in both ischaemic and non-ischaemic cardiomyopathy [Pitkin *et al* 2010b]. The reduction in receptor density in the absence of changes in expression in ischaemic heart failure may reflect alterations in post-transcriptional processing or, alternatively, greater internalisation of the receptor due to increased ligand activation. Apelin expression is increased in failing human left ventricle irrespective of aetiology whilst protein levels are unchanged [Földes *et al* 2003]. Interestingly, myocardial expression of the apelin receptor gene and cardiac apelin tissue concentrations were markedly increased following offloading of the ventricle by implantation of a left ventricular assist device [Chen *et al* 2003].

1.8.3 EFFECTS OF EXOGENOUS APELIN ADMINISTRATION IN PRECLINICAL MODELS OF HEART FAILURE

The ability of apelin peptides to enhance contractility in healthy myocardium whilst simultaneously reducing loading conditions suggests potential therapeutic application in heart failure. Crucially, these effects are maintained and possibly amplified in the failing heart. Apelin increases contractility *in vitro* to the same or even greater extent in failing myocardium as it does in normal myocardium [Dai *et al* 2006; Farkasfalvi *et al* 2007]. *In vivo*, in rats with established heart failure post myocardial infarction, apelin infusion restores ejection fraction, increases

cardiac output and reduces LVEDP [Berry *et al* 2004; Atluri *et al* 2007]. Furthermore, in a rat model of severe heart failure that exhibits features of cardiogenic shock, apelin improved cardiac contractility, loading conditions and blood pressure despite a reduction in myocardial apelin receptor expression of over 50% [Jia *et al* 2006]. These findings suggest that the signalling capacity of cardiac apelin receptors is not exhausted even when endogenous apelin receptor expression is diminished.

The beneficial effects of exogenous apelin administration may extend beyond improving cardiac performance in established heart failure to affording cardioprotection during myocardial injury. In the isoprotenerol heart failure model outlined above, concurrent administration of apelin preserved cardiac function and reduced indices of myocardial injury, preventing the development of heart failure [Jia *et al* 2006]. Moreover, in rat models of myocardial infarction, bolus infusion of apelin at the time of reperfusion, reduced infarct size *in vitro* and *in vivo* by 39% and 43% respectively [Simpkin *et al* 2007]. The reduction in infarct size was associated with activation of components of the reperfusion injury salvage kinase pathway, a key cell-signalling system that protects against ischaemia-reperfusion injury [Hausenloy *et al* 2004]. In light of this, the aforementioned up regulation of apelin expression in response to ischaemia may serve as an endogenous cardioprotective mechanism to limit myocardial injury during ischaemia.

1.8.4 POTENTIAL THERAPEUTIC ROLE OF APELIN RECEPTOR AGONISM IN PATIENTS WITH HEART FAILURE

In preclinical models, exogenous apelin reduces ventricular preload and afterload and increases myocardial contractility whilst endogenous apelin maintains cardiac performance during ageing and pressure overload. Rodents with heart failure exhibit down regulation of the apelin pathway that parallels the decline in cardiac function, yet the favourable haemodynamic effects of exogenous apelin are maintained. Together, these data suggest that augmentation of apelin signalling holds major promise for the treatment of heart failure, particularly as the apelin system undergoes a similar down regulation in patients with advanced chronic heart failure.

The first tentative evidence that disturbed endogenous apelin signalling may have functional relevance in human heart failure was recently provided in a cohort of patients with dilated cardiomyopathy [Sarzani *et al* 2007]. In these patients, possession of a polymorphism of the apelin receptor, 212A (the biological significance of which is unknown) was associated with slower progression of heart failure. Notably, no difference in the frequency of this polymorphism was noted between dilated cardiomyopathy patients and controls, suggesting that the pathophysiological significance of apelin signalling in heart failure may not relate to causation of heart failure *per se*, but rather in modulating the progression of myocardial dysfunction.

However, to date, there are no data on the cardiovascular effects of apelin *in vivo* in man. Detailed clinical investigation is therefore required to establish the role of

apelin in human cardiovascular physiology and pathophysiology, and to determine the therapeutic potential of apelin receptor agonism in patients with heart failure.

1.9 HYPOTHESES

In a series of *in vivo* clinical studies, I will address the following hypotheses:

In healthy volunteers:

1. Apelin receptor agonism causes vasodilatation in dorsal hand veins and forearm resistance vessels via the endothelial release of nitric oxide and prostacyclin.
2. Systemic apelin receptor agonism increases cardiac output whilst reducing peripheral vascular resistance and blood pressure.
3. Apelin-36 elicits more sustained cardiovascular effects than (Pyr^{1})apelin-13.

In patients attending for diagnostic coronary angiography without obstructive coronary artery disease:

1. Intracoronary apelin administration increases coronary blood flow and left ventricular contractility.

In patients with chronic heart failure:

1. Direct vascular and systemic haemodynamic responses to apelin are diminished compared with age-matched controls.

1.10 AIMS

The aims of this thesis were:

In healthy volunteers (Chapter 3):

- To establish the safety and tolerability of local intra-arterial and intravenous infusion of apelin peptides.
- To determine the direct *in vivo* actions of acute apelin receptor agonism on peripheral venous and resistance vessel tone.
- To compare the direct vascular actions of apelin-36 and (Pyr^{1})apelin-13.

In patients attending for diagnostic coronary angiography (Chapters 3 and 5):

- To establish the direct actions of apelin receptor agonism on coronary blood flow and left ventricular contractility.

In healthy volunteers (Chapter 4):

- To characterise the onset, offset, reproducibility and sustainability of vasomotor responses to apelin-36 and (Pyr^{1})apelin-13.
- To determine the contribution of the endothelium-derived vasodilators, nitric oxide and prostacyclin, to apelin-mediated vasodilatation.

In healthy volunteers (Chapter 5):

- To establish the effects of systemic apelin receptor agonism on cardiac output, peripheral vascular resistance blood pressure and heart rate.

- To compare the systemic haemodynamic effects of apelin-36 and (Pyr^{1})apelin-13.

In patients with stable chronic heart failure (Chapter 6):

- To compare the direct vascular, systemic haemodynamic and neurohormonal responses to acute apelin receptor agonism with age- and sex-matched healthy control subjects.

CHAPTER 2

METHODS

2.1 INTRODUCTION

Previous studies of apelin peptides have been carried out *in vitro* and in animal models but their effects have not been previously studied *in vivo* in man. Combined with local drug infusions, the Aellig hand vein technique and bilateral forearm venous occlusion plethysmography are ideal methods for assessing the direct vascular effects of novel agents *in vivo* as the use of subsystemic doses minimises the risk of harmful effects and avoids potentially confounding effects on other organs such as the heart and kidney. Similarly, intracoronary drug administration permits assessment of the direct actions of novel agents on the heart and coronary circulation. Regional infusion studies can then be followed by systemic administration to allow assessment of the net effects on systemic haemodynamics. An overview of the techniques employed in this thesis is presented below. Details specific to each study can be found in the methods section of subsequent chapters.

2.1.1 AELLIG HAND VEIN TECHNIQUE

The Aellig hand vein technique is a safe and reproducible method for assessing direct pharmacological effects on venous tone *in vivo* [Aellig 1981; Aellig 1994a; Aellig 1994b]. A linear variable differential transformer (LVDT) is used to measure changes in the diameter of a single dorsal hand vein at a standardised congestion pressure during local drug infusion. When the venous pressure remains constant, changes in venous diameter are proportional to changes in venous tone. Thus *in vivo* venomotor responses to infused drugs can be studied in a single vein at locally active doses without confounding systemic influences.

2.1.2 FOREARM VENOUS OCCLUSION PLETHYSMOGRAPHY

Bilateral forearm venous occlusion plethysmography, combined with unilateral intrabrachial drug infusion is an extensively validated, accurate and reproducible method of directly assessing the local effects of vasoactive agents *in vivo* [Walker *et al* 2001; Wilkinson and Webb 2001]. When examining *in vivo* vascular responses in man, systemic drug administration causes concomitant effects on organs, such as the brain, kidney and heart, and influences neurohumoral reflexes through changes in systemic haemodynamics. Because of these confounding influences, vascular responses cannot be wholly attributed to a direct effect of the drug. However, forearm vascular responses to intrabrachial drug infusion can be studied at doses 10-1000-fold lower than those required to elicit a systemic effect. This reduction in dose not only avoids confounding systemic effects but minimises the potential for side effects and toxicity.

Cuffs secured around the upper arms are intermittently inflated to above venous pressure but below diastolic pressure. During cuff inflation, venous drainage from the forearm is prevented whilst arterial inflow is unaltered. This results in a linear increase in forearm volume over time, proportional to arterial blood flow, that is detected by mercury-in-silastic strain gauges placed around the forearm. Under resting conditions, approximately 70% of total forearm blood flow (FBF) is through skeletal muscle and alterations in blood flow (assuming no change in cardiac output) predominantly reflect changes in peripheral resistance vessel tone. As the hand contains a high proportion of arteriovenous shunts and because skin blood flow is

highly dependent on temperature, the hands are excluded from the circulation during measurements by inflation of cuffs, placed around the wrists, to suprasystolic pressure. The non-infused arm serves as a contemporaneous control during studies, allowing changes in infused arm blood flow to be attributed to local vascular effects rather than changes in systemic haemodynamics.

2.1.3 CORONARY BLOOD FLOW AND LEFT VENTRICULAR PRESSURES

Although concordance between vasomotor responses in the forearm and other vascular beds is generally good, differences do exist [Hirooka *et al* 1994; Wilkinson and Webb 2001] and findings cannot be extrapolated to the coronary arterial circulation. Intracoronary drug administration with simultaneous measurement of coronary blood flow allows assessment of the direct vascular effects on the coronary circulation [Newby 2000]. Similar to the Aellig technique and forearm venous occlusion plethysmography, local intracoronary injection has the advantage of assessing the heart and coronary circulation in relative isolation without inducing systemic effects. In addition, relatively high doses can be administered locally which may be necessary to achieve the desired physiological or therapeutic effect. Measurement of coronary blood flow velocity can be achieved with a Doppler flow wire, a miniature Doppler ultrasound crystal mounted on the tip of a 0.014-inch guide wire [Doucette *et al* 1992]. In addition, cross-sectional area of the coronary vessel can be estimated using quantitative coronary angiography (QCA) allowing calculation of coronary blood flow [De Feyter *et al* 1991; Newby 2000]. Bolus injection of drugs enables precise and selective coronary administration. The injection itself causes an instantaneous increase in blood flow velocity, due to flow-

mediated dilatation. However this effect is transient and can usually be distinguished from true drug responses by avoiding prolonged injections or large volume boluses and comparing drug effects with control saline injections [Newby 2000].

Direct measurement of left ventricular pressures during drug administration permits assessment of both cardiac loading conditions and the force of left ventricular systolic contraction. This can be achieved by placement of a pressure wire within the left ventricular cavity [Duckett *et al* 2011; Ginks *et al* 2011]. The pressure wire is a fine-calibre (0.014-inch) high-fidelity wire that has a torque similar to an angioplasty wire [Blows and Redwood 2007]. There is a pressure sensor located 30 mm from its tip that can be used to record intraventricular pressure with a frequency response of 400 Hz. Continuous measurement of left ventricular pressure over the cardiac cycle allows calculation of dP/dtmax during isovolumic contraction, a measure of the force of left ventricular contraction [Rahimtoola 1973]. In addition, LVEDP and maximum left ventricular systolic pressure (LV max) serve as indices of preload and afterload respectively. As dP/dtmax is sensitive to changes in preload, afterload and heart rate, it does not provide an index of intrinsic myocardial contractility. However this limitation can, to some extent be overcome by direct intracoronary drug administration which serves to minimise systemic vascular changes.

2.1.4 THORACIC ELECTRICAL BIOIMPEDANCE

Thoracic electrical bioimpedance (TEB) cardiography is a non-invasive method of assessing cardiac output [Woltjer *et al* 1997]. The technique uses changes in thoracic electrical impedance to measure changes in the duration of cardiac ejection, and the

velocity of blood flow within the aorta. Four dual sensors are placed on either side of the neck and thorax. The outer sensors transmit a constant, low-amplitude alternating electrical current to the thorax. The corresponding voltage is detected by the inner sensors and used to determine thoracic impedance, according to Ohm's law. After filtering of the respiratory component, changes in impedance predominantly reflect changes in the volume and velocity of blood in the aorta during systole and diastole.

Thoracic electrical bioimpedence has been validated against thermodilution measurements with correlation coefficients ranging from 0.83-0.90 and mean differences ranging from 2-12% [Appel *et al* 1986; Salandin *et al* 1988; Northridge *et al* 1990; Thomas 1992]. In addition, measures of changes in cardiac output after drug intervention are in close agreement with simultaneous thermodilution measurements [Thomas 1992]. The safety and reproducibility of the bioimpedance technique makes it a suitable method for studies of drug action [Haynes *et al* 1993].

2.2 STUDY PARTICIPANTS AND CONDITIONS

2.2.1 ETHICAL CONSIDERATIONS

All studies were performed with the approval of the local Research Ethics Committee, in accordance with the Declaration of Helsinki, and with the written informed consent of all subjects.

2.2.2 STUDY PARTICIPANTS

Healthy volunteers and control subjects were not taking any regular medication and had no history of any clinically significant medical condition or symptoms of recent infective or inflammatory illness.

Patients attending for diagnostic angiography were excluded if they were found to have left main stem stenosis or severe coronary artery stenosis, previous coronary intervention, and clinical or echocardiographic evidence of cardiac failure.

Patients with heart failure were eligible for inclusion if they had stable NYHA class II–IV symptoms, were on maximally tolerated doses of heart failure medication for at least 3 months, and had objective evidence of left ventricular impairment (left ventricular end-diastolic diameter >5.5 cm and left ventricular ejection fraction <40% or shortening fraction <20%). Patients were excluded if they had haemodynamically significant valvular heart disease, renal or hepatic failure, or had previous malignant ventricular arrhythmias.

2.2.3 SUBJECT PREPARATION

All studies were carried out in a quiet, temperature-controlled room (22-25°C). Participants were semi-recumbent (venous studies) or supine and had abstained from alcohol for 24 hours, and from food and caffeine-containing drinks for at least 4 hours before the study. Healthy volunteers and controls avoided vasoactive and non-steroidal anti-inflammatory drugs for 7 days prior to studies and patients with

heart failure withheld their usual medications on the day of the study until completion of the study protocol.

2.2.4 BLOOD PRESSURE AND HEART RATE MONITORING

Heart rate and blood pressure were monitored continuously by invasive manometry during intracoronary studies and at regular intervals throughout all other studies with a semi-automated, non-invasive oscillometric sphygmomanometer (HEM 705CP; Omron, Tokyo, Japan).

2.3 DRUGS AND MATERIALS

2.3.1 DRUGS

The effects of apelin agonism were assessed using synthetic pharmaceutical grade apelin-36 and (Pyr^{1})apelin-13 (Clinalfa AG, Läufelfingen, Switzerland). Biological activity of the peptides was established by confirmation of depressor responses in rats (n=4; data on file).

Preconstriction of dorsal hand veins was achieved by norepinephrine (Hospira, IL, USA). Sodium nitroprusside (SNP; Mayne Pharma Plc, Warwickshire, UK), glyceryl trinitrate (GTN; UCB Pharma, Slough, UK) and acetylcholine (Novartis Pharmaceuticals UK Ltd, Frimley, UK) were employed in regional infusion studies as control vasodilators. Endogenous nitric oxide production was inhibited with L-N^{G}-monomethyl-arginine (L-NMMA; Clinalfa AG, Läufelfingen, Switzerland).

Cycloxygenase inhibition was achieved by a single 600 mg dose of dispersible aspirin (Aspar Pharmaceuticals Ltd, London, UK), dissolved in carbonated water.

All infused drugs were administered following dissolution in 0.9% physiological saline (Baxter Healthcare Ltd, Thetford, Norfolk, UK) under aseptic conditions on the day of the study.

2.4 DORSAL HAND VEIN STUDIES

2.4.1 INTRAVENOUS ADMINISTRATION

A 23-gauge butterfly cannula (Smiths Medical International, Watford, UK) was inserted into a non-branching dorsal hand vein of the non-dominant arm in the direction of flow and attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK). Patency was maintained by infusion of saline via an IVAC P6000 syringe pump (Alaris Products, Basingstoke, UK). The total rate of intravenous infusions was maintained constant throughout all studies at 0.25 mL/min.

2.4.2 DORSAL HAND VEIN MEASUREMENT

The hand was supported above the level of the heart by means of an arm rest and an upper arm cuff inflated to 40 mmHg to obstruct venous return [Aellig 1981] as shown in Figure 2.1. A magnetised lightweight rod was rested on the summit of the infused vein, 1 cm downstream from the tip of the infusion needle. The rod was passed through the core of a LVDT (Model 025 MHR, Lucas Schaevitz Inc, Pennsauken, NJ, USA), supported above the hand by a small tripod (Figure 2.1).

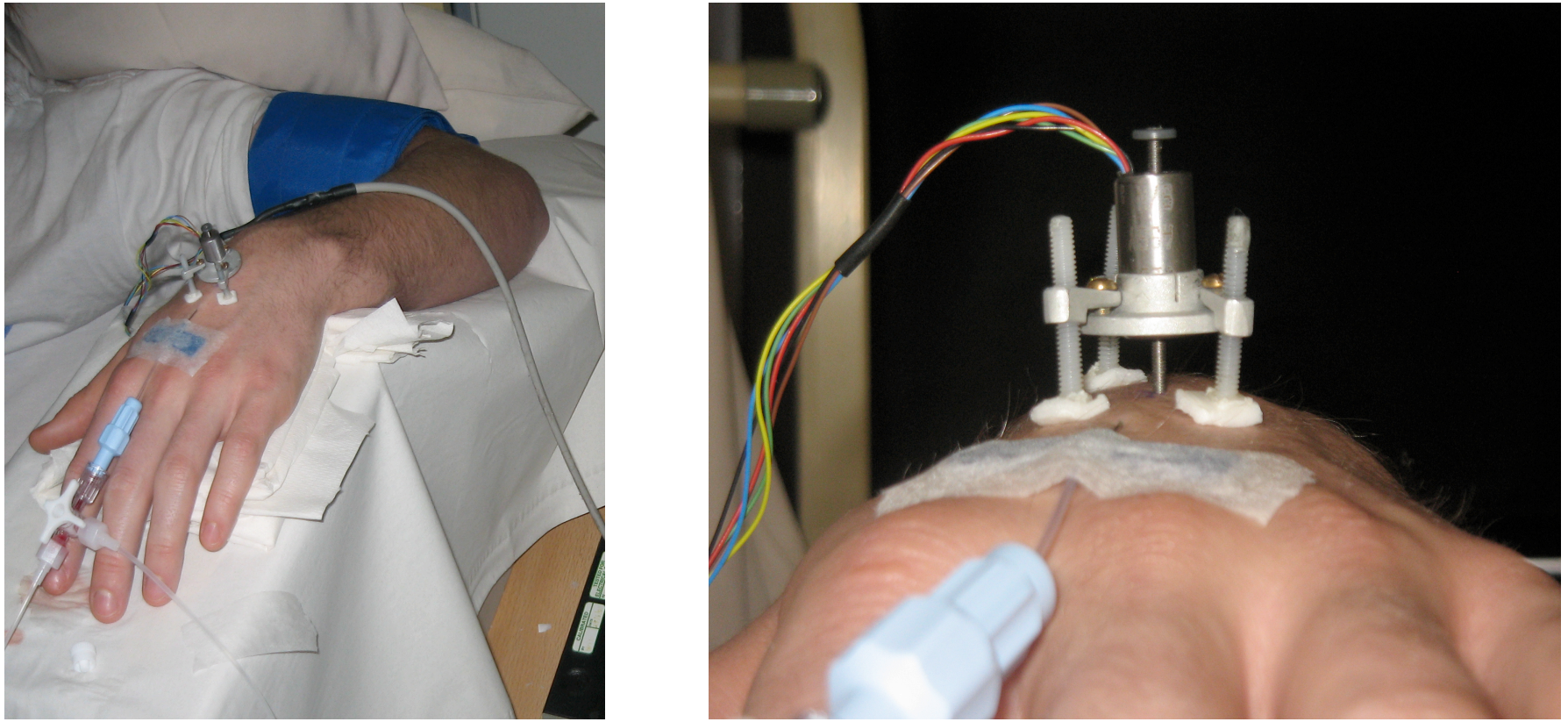

Figure 2.1 Aellig dorsal hand vein technique

Vertical displacement of the rod following transient cuff deflation causes a linear change in the voltage generated by the LVDT reflecting changes in the internal diameter of the vein. The voltage output from the LVDT was transferred to a Macintosh Classic II computer (Apple Computer Inc, Cupertino, CA, USA) using a MacLab analogue-to-digital converter (AD Instruments Ltd, Castle Hill, NSW, Australia) and Chart™ software (Version 5.4.2, AD Instruments).

2.4.3 DATA ANALYSIS

Baseline vein diameter was calculated as the mean of the last three measurements during the initial infusion of saline, before the start of active drug infusion. The intra-subject coefficient of variation for between-day measurements was 10.0% for basal hand vein diameter and 15.3% for hand vein responses to SNP. In order to minimise the effects of inter-subject variability in hand vein diameter, responses to local drug infusion are expressed as percentage change in vein diameter from baseline [Haynes *et al* 1994; Gudmundsdóttir *et al* 2006].

2.5 FOREARM PLETHYSMOGRAPHY STUDIES

2.5.1 BRACHIAL ARTERY CANNULATION

Subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 2% lidocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia (Figure 2.2). The cannula was attached to a 16-gauge epidural catheter

Figure 2.2 Set-up for forearm venous occlusion plethysmography studies.

and patency maintained by infusion of saline 0.9% via an IVAC P6000 syringe pump. In all studies, saline was infused for the first 20 minutes to allow for equilibration and the intra-arterial infusion rate remained constant at 1 mL/min.

2.5.2 BLOOD FLOW MEASUREMENT

Blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges that were applied to the widest part of the forearm [Wilkinson and Webb 2001]. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (DE Hokanson Inc, WA, USA). Upper arm cuffs were inflated intermittently to 40 mmHg pressure for 10 seconds in every 15 seconds to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge plethysmograph (DE Hokanson Inc, WA, USA) was processed by a PowerLab analogue-to-digital converter and Chart™ software (version 5.0.1, AD Instruments) and recorded onto a Dell Latitude laptop computer (Dell Computers Ltd, UK). Calibration was achieved using the internal standard of the plethysmograph.

2.5.3 INHIBITION OF NITRIC OXIDE AND CYCLOOXYGENASE

Endogenous nitric oxide was inhibited by the use of a 'nitric oxide clamp', as described previously [Stroes *et al* 1997]. In brief, the nitric oxide synthase inhibitor L-NMMA was continuously infused (8 μmol/min) to achieve maximal inhibition of local nitric oxide synthesis [Vallance *et al* 1989; Calver *et al* 1992; Stroes *et al* 1995]. After 20 minutes of L-NMMA infusion, when steady state blood

flow was obtained, SNP, an exogenous nitric oxide donor, was co-infused in incremental doses (90-900 ng/min) and titrated to restore baseline FBF. L-NMMA and SNP were then co-infused, at these rates, for the remainder of the study; thus allowing simulation of normal basal nitric oxide activity during continuous inhibition of endogenous nitric oxide synthesis [Honing *et al* 2000; Ueda *et al* 2004].

Inhibition of the cyclooxygenase enzyme and thus the production of prostaglandins and thromboxanes was achieved by a single dose of dispersible aspirin (600 mg), dissolved in carbonated water and administered orally 30 minutes before study commencement. This dose of aspirin rapidly inhibits bradykinin-stimulated endothelial production of prostacyclin by at least 85% with recovery developing over the next 6 hours [Heavey *et al* 1985].

2.5.4 DATA ANALYSIS

Plethysmographic data were extracted from the Chart™ data files (Figure 2.3) and FBF was calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Microsoft Excel for Mac 2004; Microsoft Corporation, WA, USA). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the variability in blood flow that this incurs [Benjamin *et al* 1995]. Usually, the last five flow recordings in each 3-minute measurement period were calculated and averaged for each arm. The non-infused arm acted as a contemporaneous control for systemic effects and external influences upon FBF.

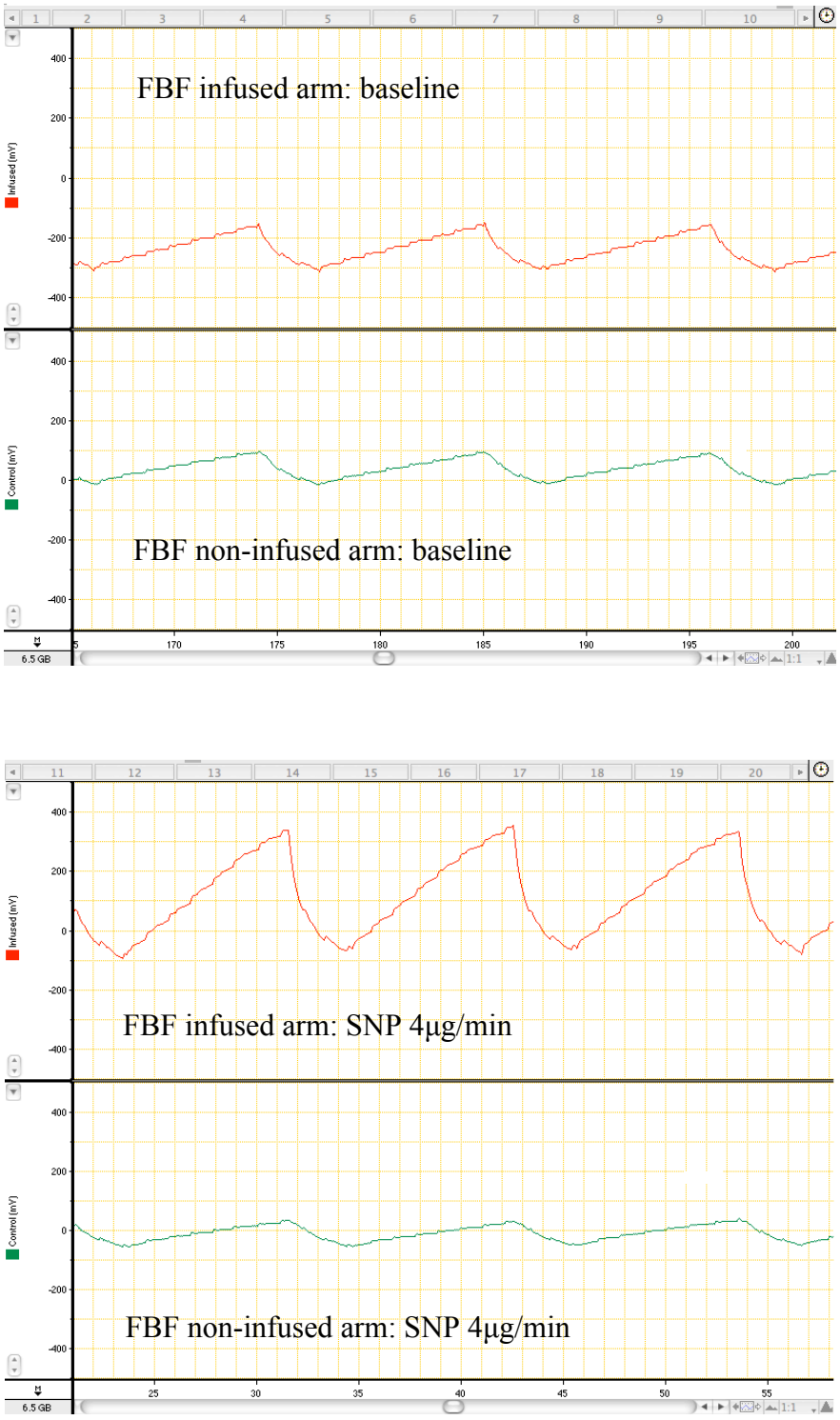

Figure 2.3 Typical plethysmographic traces

The effect of the nitric oxide clamp and aspirin on FBF responses to (Pyr^{1})apelin-13 and acetylcholine were assessed by calculating area under the curve (AUC) during drug infusions using the trapezoid method.

Analysis of all data collected during the forearm plethysmography study was undertaken by a single operator in a blinded fashion. Forearm blood flow responses are reported as absolute blood flow responses (mL/100 mL tissue/min) in the infused and non-infused arm. The intra-subject coefficient of variation for between-day measurements was 11.7% for baseline FBF and 16.3% for blood flow responses to apelin-36.

2.6 CORONARY AND CARDIAC STUDIES

2.6.1 CARDIAC CATHETERISATION

Following diagnostic coronary angiography and intravenous administration of unfractionated heparin (5000 units), a coronary guide catheter was engaged in the ostium of the left coronary artery and a 0.014-inch 12.5 MHz Doppler wire (FloWire, Cardiometrics Inc, Mountain View, CA, USA) positioned in the coronary artery to measure blood flow velocity. A further catheter was inserted into the left ventricle and a 0.014-inch pressure wire (RADI systems, Uppsala, Sweden) placed in the left ventricular cavity.

2.6.2 MEASUREMENT OF CORONARY BLOOD FLOW

Coronary angiography was performed, immediately prior to, and 30 seconds after, each drug bolus. Blood flow velocity was determined using the average peak velocity of the Doppler signal [Newby *et al* 2001].

2.6.3 MEASUREMENT OF LEFT VENTRICULAR PRESSURE

The output from the pressure wire was connected to a Radi analyzer® and connected to a computer with PhysioMon® software to give curves showing real-time blood pressure and left ventricular dP/dtmax.

2.6.4 DATA ANALYSIS

Coronary luminal diameter was measured by quantitative computerised analysis with an automated edge contour detection analysis system from end-diastolic frames of each angiogram and cross-sectional area calculated assuming circular geometry [Newby *et al* 2001]. The stem of the coronary catheter was used for calibration to determine absolute measurement in millimetres, and correction. Coronary blood flow was defined as half the product of the average peak velocity and the cross-sectional area of the coronary artery [Newby *et al* 2001]. Ten-second samples of pressure recordings within the left ventricle were taken before and at 60 seconds after each drug bolus and used to calculate dP/dtmax, LV max and LVEDP. For within-day measurements, the intra-subject coefficient of variation was 13.0% for coronary blood flow and 4.0% for dP/dtmax.

2.7 SYSTEMIC HAEMODYNAMIC STUDIES

2.7.1 INTRAVENOUS ADMINISTRATION

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms to allow drug infusion and sampling of venous blood. The rate of infusion was kept constant at 1 mL/min throughout all studies.

2.7.2 MEASUREMENT OF SYSTEMIC HAEMODYNAMIC VARIABLES

Cardiac output was measured using a TEB monitor (Modular HOTMAN® System with EXT-TEBCO® Module, HemoSapiens Inc, CA, USA), following manufacturer's guidelines. The EXT-TEBCO® Module was attached via a USB cable to a Dell Latitude laptop computer (Dell Computers Ltd, UK) installed with HOTMAN® software. After preparing the skin with alcohol to to ensure good adhesion and low skin-to-electrode impedance, EXT-TEBCO® Module was connected to the patients via four pairs of low contact impedance electrodes [Northridge *et al* 1990] (Figure 2.4). The lower thoracic voltage sensing electrodes placed at the level of the xiphoid sternum in the midaxillary lines and the cervical sensing electrodes were positioned laterally at the base of the neck as close as possible to the clavicles. The 'current injecting' electrodes delivered an alternating current of 7 μA at 65 kHz and were placed with one pair 5 cm above the cervical sensing electrodes and the other pair 5 cm below the thoracic sensing electrodes.

Figure 2.4 TEBCO module and electrodes connected to laptop computer.

The EXT-TEBCO® Module estimated stroke volume from the impedance signal recorded from the inner pairs of electrodes using the Sramek-Bernstein equation [Bernstein *et al* 1986].

$$\mathbf{SV = VEPT \times VET \times (dz/dt)max/ZO}$$

Where VEPT=volume of electrically participating tissue (a constant derived from body height and weight); VET=ventricular ejection time; dz/dt=rate of change of impedance during systole; and ZO=basal thoracic impedance.

The monitor automatically averages cardiac output over 16 cardiac cycles and displays it continuously in real-time. Blood pressure and heart rate were measured periodically with a semi-automated non-invasive oscillometric sphygmomanometer (HEM 705CP; Omron, Tokyo, Japan) and values entered manually via the laptop keyboard.

2.7.3 DATA ANALYSIS

At each time point, cardiac output was recorded manually from the continuous monitor display as the mean of three recordings, each recording representing the average of 16 consecutive heartbeats [Northridge *et al* 1990]. Cardiac output was corrected for body surface area to give cardiac index. Mean arterial pressure was defined as the sum of the diastolic blood pressure and a third of the pulse pressure. Peripheral vascular resistance index was calculated as mean arterial pressure divided by cardiac index and expressed in dyne.s/cm^5/m^2. For between-day measurements of cardiac output, the coefficient of variation was 6.4%.

2.8 BLOOD SAMPLING AND ASSAYS

Blood samples (10 mL) were drawn by a single venepuncture or repeatedly via a venous cannulae (17-gauge) inserted into a large subcutaneous vein of the antecubital fossa into tubes containing either ethylene diamine tetraacetic acid (EDTA) or lithium heparin, and kept on ice before centrifugation at 2000 *g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay.

Plasma apelin-36 concentration was measured using the apelin-12 micro-plate enzyme-linked immunosorbent assay (ELISA) kit (Phoenix Europe GmbH, Karlsuhe, Germany). The antibody used in this assay has 100% cross-reaction with apelin-12, apelin-13 and apelin-36 but cross-reaction with (Pyr^{1})apelin-13 is unknown. The minimal detectable apelin concentration with this assay is 0.07 ng/mL. The intra-assay coefficient of variation was 9% and the inter-assay coefficient of variation <15%.

Plasma renin activity was measured under standard conditions through the generation of angiotensin I as determined by radioimmunoassay (DiaSorin Ltd, Wokingham, UK) [Haber *et al* 1969]. Plasma brain natriuretic peptide (BNP) and AVP concentrations were measured by immunoradiometric assay (Shionogi and Co Ltd, Osaka, Japan) and direct radioimmunoassay (Bühlmann Laboratories AG, Schonebuch, Switzerland) respectively. The intra- and inter-assay coefficients of

variation for plasma renin activity, BNP and AVP were 7.3% and 6.7%; 2.7% and 4.2%; and 6.0% and 9.9% respectively.

2.9 STATISTICS

Data were examined by analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test as appropriate using GraphPad Prism (GraphPad Software, Inc, CA, USA). All results are expressed as mean ± standard error of the mean (SEM). Statistical significance was taken at the 5% level.

CHAPTER 3

DIRECT VASCULAR ACTIONS OF APELIN

IN VIVO IN MAN

Japp AG, Cruden NL, Amer DA *et al*.
Vascular effects of apelin in vivo in man.
J Am Coll Cardiol 2008; **52**:908-913.

3.1 SUMMARY

In preclinical models, apelin causes arterial and venous vasodilatation and is the most potent inotrope yet described. We aimed to establish the direct *in vivo* vascular effects of apelin in man. Vascular effects of apelin were assessed in 14 healthy volunteers and 6 patients undergoing diagnostic coronary angiography. Dorsal hand vein diameter was measured by the Aellig technique during local intravenous infusions of apelin-36 and (Pyr^1)apelin-13 (0.1-3 nmol/min) and SNP (0.6 nmol/min). Forearm blood flow was measured by venous occlusion plethysmography during intrabrachial infusions of apelin-36 and (Pyr^1)apelin-13 (0.1-10 nmol/min). Coronary blood flow was measured by Doppler flow wire and QCA following intracoronary boluses of apelin-36 (20 and 200 nmol in 2 mL), 0.9% saline (2 x 2 mL) and GTN (100 μg in 2 mL). Although SNP caused venodilatation ($P<0.0001$), apelin-36 and (Pyr^1)apelin-13 had no effect on dorsal hand vein diameter ($P=0.2$). Both apelin-36 and (Pyr^1)apelin-13 caused vasodilatation in forearm resistance vessels ($P<0.0001$). Intracoronary bolus of apelin-36 increased coronary blood flow ($P<0.05$). Although having no apparent effect on venous tone, acute apelin administration in man causes peripheral and coronary vasodilatation. The apelin system merits further clinical investigation to determine its role in cardiovascular homeostasis and its therapeutic potential in heart failure.

3.2 INTRODUCTION

Apelin was identified in 1998 [Tatemoto *et al* 1998] as the endogenous ligand for the then orphan G-protein coupled receptor, APJ [O'Dowd *et al* 1993], now renamed the 'apelin receptor' [Pitkin *et al* 2010a]. The apelin gene encodes a 77 amino acid prepropeptide that undergoes proteolytic cleavage to yield bioactive peptides of variable length: 13, 17 and 36 amino acids [Tatemoto *et al* 1998; Habata *et al* 1999; Hosoya *et al* 2000]. The shorter isoforms have greater affinity for the apelin receptor [Hosoya *et al* 2000; Masri et al 2006] and more potent cardiovascular effects in preclinical models [Tatemoto *et al* 2001], particularly the pyroglutamated form of apelin-13, (Pyr^{1})apelin-13. Widely expressed in the CNS and peripheral tissues [De Mota *et al* 2000; Hosoya *et al* 2000; Kawatama *et al* 2001; De Falco *et al* 2002], the apelin system participates in a diverse array of processes including glucose metabolism [Sorhede *et al* 2005], food intake [Sunter *et al* 2003], thermoregulation [Jaszberenyi *et al* 2004] and fluid balance [De Mota *et al* 2004]. However, its principal physiological role appears to relate to its cardiovascular actions.

Apelin receptors are present on endothelial cells and vascular smooth muscle cells [Kleinz *et al* 2005], and in preclinical models, apelin signalling exerts major effects on vascular tone. In rodent models, exogenous apelin administration causes a rapid fall in arterial blood pressure [Reaux *et al* 2001; Tatemeoto *et al* 2001; El Messari *et al* 2004; Ishida *et al* 2004; Lee *et al* 2005] mediated by nitric oxide [Tatemoto *et al* 2001] and a concomitant reduction in mean capillary filling pressure [Cheng *et al* 2003], indicating powerful vasodilator and venodilator effects. To date, there have

been no *in vivo* clinical studies but *ex vivo* myography studies report that apelin causes a nitric oxide-dependent vasorelaxation in human mesenteric artery [Salcedo *et al* 2007] and venoconstriction in endothelium-denuded human saphenous vein [Katugampola *et al* 2001].

Apelin is emerging as an important mediator of cardiovascular homeostasis, yet its cardiovascular actions *in vivo* in man are unknown. Detailed clinical investigation to establish the role of the apelin system in human cardiovascular regulation is an essential prerequisite to elucidating its pathophysiological relevance and therapeutic potential. As a first step towards characterising the *in vivo* cardiovascular profile of apelin in man, we sought to determine its direct vasomotor effects in the peripheral venous and peripheral and coronary arterial circulation.

3.3 METHODS

3.3.1 SUBJECTS

Fourteen healthy volunteers aged between 19 and 24 years and six patients attending for elective outpatient diagnostic coronary angiography participated in these studies, which were performed with the approval of the local Research Ethics Committee, in accordance with the Declaration of Helsinki, and with the written informed consent of all subjects. Healthy volunteers were not taking any regular medication and had no history of any clinically significant medical condition or symptoms of recent infective or inflammatory illness. Patients attending for diagnostic angiography were excluded if they were found to have left main stem stenosis or severe coronary artery

stenosis, previous coronary intervention, and clinical or echocardiographic evidence of cardiac failure. Patients withheld their usual medications on the day of the study until completion of the study protocol and all participants abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least 4 hours before each study.

3.3.2 DRUGS

The effects of apelin receptor agonism were assessed using synthetic pharmaceutical grade apelin-36 and (Pyr^{1})apelin-13. The doses of apelin peptides were chosen to achieve end-organ concentrations equivalent to systemic concentrations generated in preclinical *in vivo* studies (assuming a dorsal hand vein flow of 1-5 mL/min, brachial artery blood flow of 25 mL/min and coronary artery blood flow of 125 mL/min) and based on preliminary exploratory forearm plethysmography studies employing doses from 0.0001-10 nmol/min (n=6; data not shown).

3.3.3 MEASUREMENTS

All studies were carried out in a quiet, temperature-controlled room (22-25°C). Participants were semi-recumbent (venous studies) or supine (peripheral and coronary arterial studies) Heart rate and blood pressure were monitored at regular intervals throughout peripheral venous and arterial studies with a semi-automated, non-invasive oscillometric sphygmomanometer, and continuously during intracoronary studies.

Venous Studies

A 23-gauge needle was sited in a non-branching dorsal hand vein in the direction of flow and the total infusion rate was kept constant at 0.25 mL/min. The hand was supported above the level of the heart and an upper arm cuff inflated to 40 mmHg to obstruct venous return. The internal diameter of the dorsal hand vein was measured by the Aellig technique [Aellig 1981]. Briefly, a magnetised lightweight rod was rested on the summit of the infused vein, 1 cm downstream from the tip of the infusion needle. The rod was passed through the core of a LVDT, supported above the hand by a small tripod. Changes in vein diameter caused vertical displacement of the rod, leading to a linear change in the voltage generated by the LVDT, thus enabling calculation of absolute changes in vein size.

Peripheral Arterial Studies

Subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions. In all studies, saline was infused for the first 20 minutes to allow for equilibration and the intra-arterial infusion rate remained constant at 1 mL/min. Forearm blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [Newby et al 1997a; Newby *et al* 1999].

Coronary Artery Studies

Following diagnostic coronary angiography, a coronary guide catheter was engaged in the ostium of the left coronary artery and a 0.014-inch 12.5 MHz Doppler wire positioned in the coronary artery to measure blood flow velocity. Additionally, as

described in Chapter 5, a further catheter was inserted into the left ventricle and a 0.014-inch pressure wire placed in the left ventricular cavity to measure left ventricular pressures. Coronary angiography was performed, immediately prior to, and 30 seconds after, each drug bolus. Coronary luminal diameter was measured by quantitative computerised analysis with an automated edge contour detection analysis system from end-diastolic frames of each angiogram and cross-sectional area calculated assuming circular geometry [Newby *et al* 2001]. Blood flow velocity was determined using the average peak velocity of the Doppler signal [Newby *et al* 2001]. Coronary blood flow was defined as half the product of the average peak velocity and the cross-sectional area of the coronary artery [Newby *et al* 2001].

3.3.4 STUDY PROTOCOL

Protocol 1: Effects of apelin receptor agonism on venous tone

Because the dorsal hand vein does not have basal resting tone, norepinephrine (1-32 ng/min) was infused to induce and maintain a 50-70% reduction in vein diameter. Six subjects then received incremental intravenous infusions (0.1, 0.3, 1, 3 nmol/min) of either (Pyr^1)apelin-13 or apelin-36 for 8 minutes at each dose followed, after a 20-minute washout period, by a single 8-minute dose of the control venodilator, SNP at 0.6 nmol/min. To ensure that norepinephrine was not masking constrictor effects, a further three subjects received the same doses of (Pyr^1)apelin-13 and apelin-36 in the absence of norepinephrine preconstriction.

Protocol 2: Effects of apelin receptor agonism on forearm resistance vessel tone

Five subjects attended on two occasions at least one week apart and received either apelin-36 or (Pyr^{1})apelin-13 at sequential doses of 0.1, 0.3, 1, 3, and 10 nmol/min for 10 minutes at each dose followed by a single 10-minute saline washout.

Protocol 3: Coronary artery and myocardial contractility studies

In a double-blind randomised cross-over manner, intracoronary boluses of apelin-36 (20 and 200 nmol in 2 mL) and 0.9% saline (2 x 2 mL) followed by a single-blinded non-randomised bolus of GTN (100 μg in 2 mL) were administered in six subjects via the coronary guide catheter. All drug boluses were followed by a 2 mL 0.9% saline flush and 5-minute washout intervals were allowed between drugs. Coronary blood flow was calculated before and after each drug bolus as described above.

3.3.5 DATA AND STATISTICAL ANALYSES

Dorsal hand vein [Haynes *et al* 1995], forearm plethysmographic [Newby *et al* 1997a] and coronary blood flow [Newby *et al* 2001] data were analysed in a blinded fashion as described previously. Variables are reported as mean ± SEM and analysed using repeated measures ANOVA with post-hoc Bonferroni corrections and two-tailed Student's *t*-test as appropriate. Statistical significance was taken at the 5% level.

3.4 RESULTS

In all studies, apelin was well tolerated with no significant adverse events. There were no changes in heart rate or blood pressure during intravenous or intrabrachial apelin infusions.

Protocol 1: Effects of apelin on peripheral venous tone

Neither (Pyr^{1})apelin-13 nor apelin-36 caused venoconstriction in relaxed dorsal hand veins (data not shown). Following stable preconstriction with norepinephrine (Figure 3.1), dorsal hand vein diameter was unaffected by (Pyr^{1})apelin-13 or apelin-36 ($P=0.2$) but was increased by SNP**.**

Protocol 2: Effects of apelin on forearm resistance vessel tone

Both (Pyr^{1})apelin-13 and apelin-36 increased blood flow in the infused arm ($P<0.0001$; Figure 3.2). Whilst the magnitude of the vasodilator response did not differ between the two isoforms ($P>0.05$, ANOVA) blood flow appeared to plateau at doses of apelin-36 >0.1 nmol/min, but continued to increase with (Pyr^{1})apelin-13 infusion up to the peak dose of 10 nmol/min. Following 10 minutes of saline washout, blood flow was unchanged with apelin-36 but appeared to decline with (Pyr^{1})apelin-13 ($P<0.05$, ANOVA; data not shown). In the non-infused arm there was no change in blood flow during apelin-36 infusion but a small increase at the maximum dose of 10 nmol/min during (Pyr^{1})apelin-13 infusion.

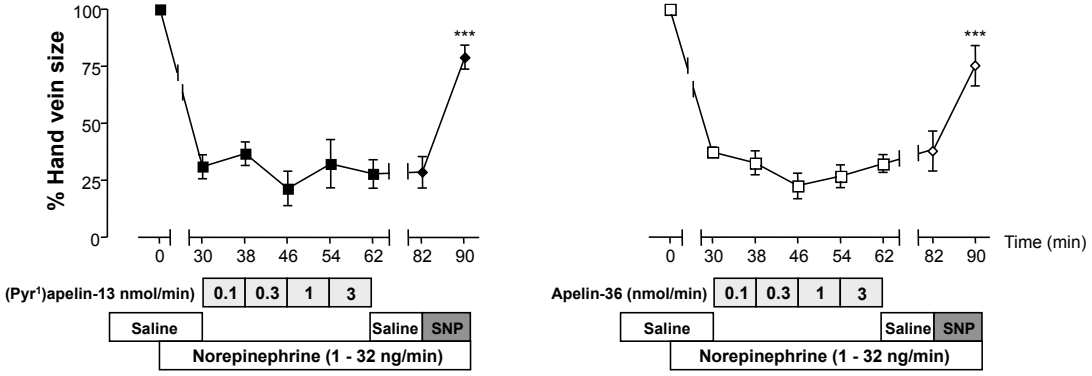

Figure 3.1 DHV diameter during co-infusion of norepinephrine and **A:** incremental doses of (Pyr^1)apelin-13 (■) and single dose of SNP (◆; 0.6 nmol/min); **B:** incremental doses of apelin-36 (□) and single dose of SNP (◇; 0.6 nmol/min). ***P<0.001, paired student's *t*-test.
DHV- dorsal hand vein; SNP - sodrium nitroprusside.

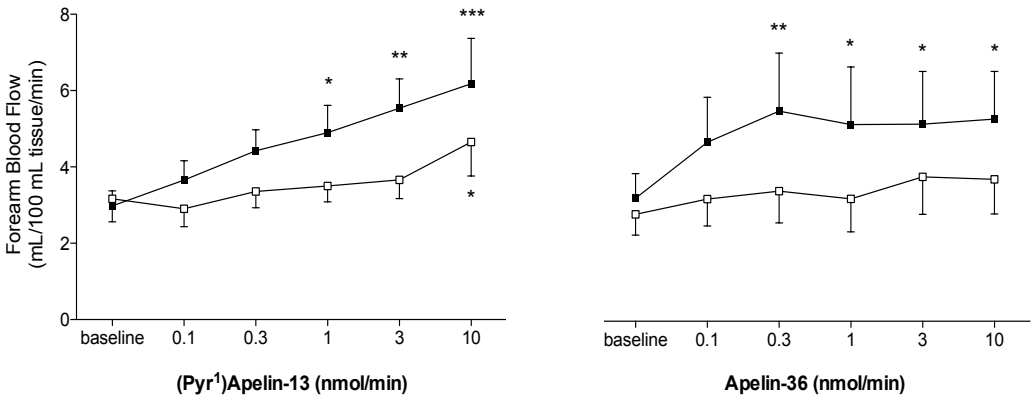

Figure 3.2 Forearm blood flow in the infused (■) and non-infused arm (□) during infusion of apelin peptides. For dose-responses to (Pyr^1)apelin-13 and apelin-36 in the infused arm: P<0.001, one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001, post-hoc Bonferroni tests *versus* baseline.
ANOVA - analysis of variance.

Coronary Blood Flow

Patients were aged 60 ± 4 years and five were male. All patients had near normal coronary arteries with no haemodynamically significant flow limiting stenoses (<25% luminal stenosis).

Left ventricular pressure measurements are reported in Chapter 5. There were no significant changes in coronary blood flow following injection of 20 nmol apelin-36 (data on file). Compared with saline placebo, there was an increase in coronary blood flow following intracoronary administration of 200 nmol apelin-36 and a trend toward an apparent increase with GTN which did not reach statistical significance (Figure 3.3). In comparison with placebo, GTN caused a fall in mean arterial pressure (94 ± 8 *versus* 80 ± 9 mmHg, P<0.01) and rise in heart rate (67 ± 5 *versus* 69 ± 5 bpm, P<0.001) but apelin had no effect on blood pressure (94 ± 8 *versus* 94 ± 7 mmHg, P=0.9) or heart rate (67 ± 5 *versus* 68 ± 5 bpm, P=0.3).

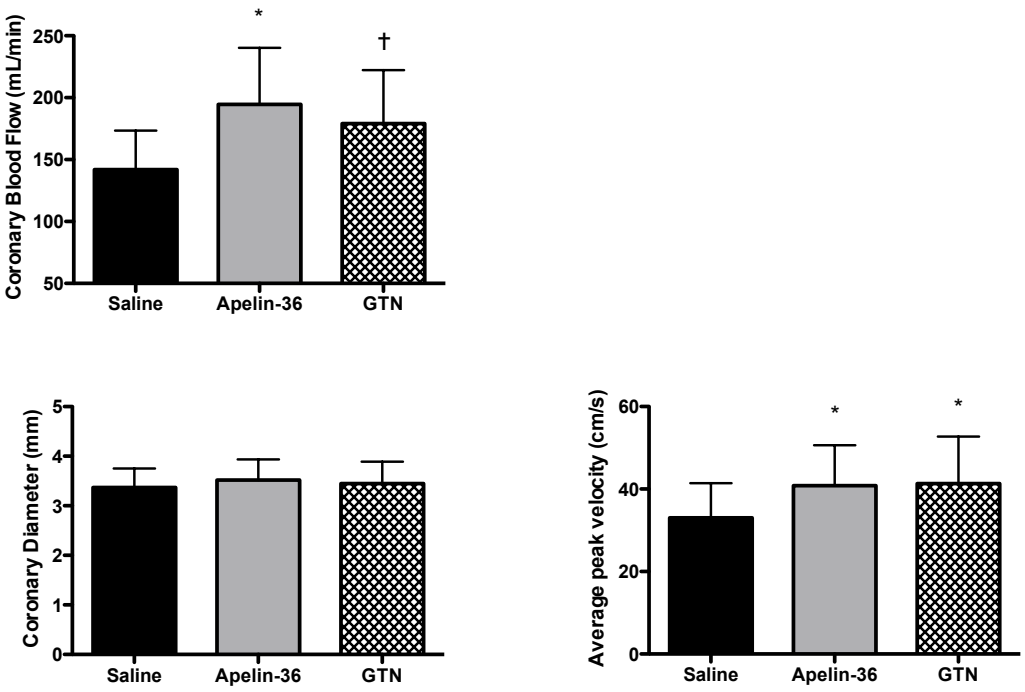

Figure 3.3 Coronary blood flow, average peak velocity of blood and epicardial coronary artery diameter following bolus injection of apelin-36 (200 nmol), GTN (100 μg) and 0.9% saline. *P<0.05, †P=0.07, paired student's *t*-test *versus* 0.9% saline.
GTN - glyceryl trinitrate.

3.5 DISCUSSION

This is the first study to examine the *in vivo* vascular actions of apelin in man. Using robust well-validated techniques, we have assessed the direct effects of two endogenous apelin peptides on vascular tone. Whilst not affecting peripheral venous tone, apelin peptides cause vasodilatation in peripheral resistance vessels and the coronary arterial circulation.

3.5.1 EFFECTS OF APELIN ON PERIPHERAL ARTERIOLAR TONE

In keeping with preclinical models and studies of human mesenteric vessels *in vitro*, we have demonstrated that apelin causes vasodilatation in human forearm resistance vessels *in vivo*. We studied two different apelin isoforms: apelin-36, the full-length mature peptide, and (Pyr^{1})apelin-13, a shorter C-terminal fragment. Although shorter apelin peptides are reported to have more potent depressor activity in rats [Tatemoto *et al* 2001], we saw no apparent difference in the overall vasodilator response to these two isoforms. However, whilst the response to (Pyr^{1})apelin-13 appeared to be dose-dependent, vasodilatation to apelin-36 plateaued from an early stage. Rapid desensitisation of responses to apelin have previously been demonstrated *in vitro*, particularly with apelin-36 [Masri *et al* 2006], most likely due to depletion of cell surface receptors following internalisation. However further dedicated *in vivo* studies will be required to distinguish this possibility from a true ceiling of vasodilator response. We measured changes in FBF during local intrabrachial apelin infusions with the non-infused arm serving as a contemporaneous control. The slight increase in blood flow in the non-infused arm

during infusion of the maximum dose of (Pyr^{1})apelin-13 raises the possibility of spillover of infused apelin into the systemic circulation. However no increase in non-infused arm blood flow was seen during apelin-36 infusion and there were no changes in heart rate or blood pressure with either apelin isoform. Measurement of systemic plasma apelin concentrations during local dose infusions would help to clarify the threshold dose for systemic spillover.

3.5.2 EFFECTS OF APELIN ON CORONARY BLOOD FLOW

In keeping with its effects in peripheral resistance vessels, we have demonstrated that apelin is a direct coronary vasodilator. Concordance between vasomotor responses in the forearm and other vascular beds is generally good [Wilkinson and Webb 2001], but differences do exist. Additionally, apelin has been shown to exert direct vasoconstrictor effects in isolated human vessels *ex vivo* [Katugampola *at al* 2001]. Given the potentially deleterious consequences of coronary vasoconstriction in conditions such as heart failure, establishing the *in vivo* effects of apelin on coronary blood flow in man is therefore an essential prerequisite to exploring its therapeutic application.

Following intracoronary administration of apelin there was an increase in coronary blood flow compared with saline placebo. The finding that the average peak velocity of blood flow increased without any concurrent change in epicardial diameter could be interpreted as indicating a predominant action of apelin on resistance rather than conductance vessels However, given the small sample size and relative lack of sensitivity of QCA [Newby *et al* 2000] for detecting small changes in lumen size, it

is perhaps likely that our study was underpowered to detect a significant change. In keeping with this we also observed no significant change in epicardial coronary diameter with GTN, an agent well known to cause dilatation of coronary conductance arteries [Hood *et al* 1980]. We employed only two doses of apelin and measured blood flow responses at a single time point following bolus injection, observing an increase only at the higher dose. Further studies will be required to characterise the dose-response relationship and time course of apelin-mediated coronary vasodilatation. Another important limitation of this study is that all of our subjects were free of significant obstructive coronary artery disease. Other endothelium-dependent vasodilators such as acetlychoine have previously been shown to exert paradoxical vasoconstriction in coronary arteries with a heavy atherosclerotic burden [Ludmer *et al* 1986]. Like acetylcholine, apelin causes vasconstriction in *ex vivo* myography studies when vessels are denuded of endothelium [Katugampola *et al* 2001]. The direct coronary effects of apelin in patients with significant coronary artery disease and other conditions associated with endothelial dysfunction will therefore require further study.

3.5.3 EFFECTS OF APELIN ON PERIPHERAL VENOUS TONE

Preclinical studies examining the venous effects of apelin have produced conflicting results. Apelin-13 lowered systemic venous tone in rats suggesting a powerful venodilator effect [Cheng *et al* 2003], but the same apelin fragment caused potent venoconstriction in *ex vivo* endothelium-denuded human saphenous vein. [Katugampola *et al* 2001]. In the present study, local *in vivo* infusion of apelin-36 or (Pyr^{1})apelin-13 had no effect on either basal or preconstricted dorsal hand vein

diameter. We demonstrated constrictor and dilator responses to norepinephrine and SNP respectively, and employed doses of apelin 10-fold higher than those required to cause vasodilatation in the forearm arterial circulation. It is therefore unlikely that the lack of venous response to apelin reflected either inadequate dosing or sensitivity of the technique. It should be noted, however, that we have examined the effects of apelin in a single peripheral venous bed. We cannot exclude the possibility that apelin may exert a direct effect on central veins. Further clinical studies are required to determine the *in vivo* effects of apelin in specific venous beds and on systemic venous tone. Finally, apelin has been shown to inhibit angiotensin II-induced venoconstriction in rat portal vein [Gurzu *et al* 2006]. While we observed no direct effect of apelin on peripheral venous tone, it remains to be determined whether apelin modulates the venomotor response to other signalling systems in man, in particular the renin-angiotensin-aldosterone system.

3.5.4 CONCLUSIONS

We have shown that acute apelin administration *in vivo* in man causes peripheral and coronary arterial vasodilation but does not appear to affect peripheral venous tone. Increasing evidence suggests that apelin mediates important effects on cardiovascular homeostasis in preclinical models, including arterial vasodilatation and increased cardiac contractility. Our findings provide the strongest evidence to date of a role for the apelin system in human cardiovascular regulation.

CHAPTER 4

CHARACTERISATION OF APELIN-MEDIATED VASODILATATION IN THE HUMAN FOREARM CIRCULATION

Japp AG, Cruden NL, Amer DA *et al.*
Vascular effects of apelin *in vivo* in man.
J Am Coll Cardiol 2008;**52**:908-913.

4.1 SUMMARY

We recently demonstrated that apelin causes coronary and peripheral arterial vasodilatation *in vivo* in man. We here aimed to characterise the vasodilator responses to two apelin peptides *in vivo* in the human forearm arterial circulation. Vascular effects of apelin were assessed in 16 healthy volunteers. Forearm blood flow was measured by venous occlusion plethysmography during intrabrachial infusions of apelin-36 and (Pyr^{1})apelin-13 (0.1-30 nmol/min) and subsequently in the presence or absence of a nitric oxide clamp (NO synthase inhibitor, L-NMMA (8 μmol/min), co-infused with SNP (90-900 ng/min)), or a single oral dose of aspirin (600 mg) or matched placebo. Both apelin-36 and (Pyr^{1})apelin-13 caused reproducible vasodilatation in forearm resistance vessels ($P<0.0001$) with a rapid onset and prolonged offset. Plasma apelin concentrations and blood flow in the non-infused arm ($P<0.05$) were increased at doses >3 nmol/min, consistent with systemic spillover. At subsystemic doses, the two apelin isoforms elicited equivalent vasodilatation. Continuous infusion of (Pyr^{1})apelin-13 for 42 minutes elicited sustained, near maximal vasodilatation. (Pyr^{1})apelin-13-mediated vasodilatation was attenuated by the nitric oxide clamp ($P=0.004$) but was unaffected by aspirin ($P=0.7$). Apelin causes nitric oxide-dependent arterial vasodilatation *in vivo* in man. The apelin system merits further clinical investigation to determine its role in cardiovascular homeostasis and its therapeutic potential in heart failure.

4.2 INTRODUCTION

Apelin is the endogenous ligand [Tatemoto *et al* 1998] for the previously 'orphan' G-protein coupled receptor, APJ [O'Dowd *et al* 1993], now renamed the 'apelin receptor' [Pitkin *et al* 2010a] The full-length mature apelin peptide comprises 36 amino acids (apelin-36) but a 13 amino acid fragment of its C-terminus has also been detected *in vivo* and exhibits biological activity [Hosoya *et al* 2000]. This fragment undergoes a pyroglutamate substitution at its N-terminus *in vivo* ((Pyr^{1})apelin-13), likely rendering it more resistant to enzymatic cleavage [Kleinz and Davenport 2005]. The distinct physiological roles of these two different apelin peptides and the principal endogenous ligand remains uncertain: (Pyr^{1})apelin-13 is the more abundant isoform in cardiac tissue and plasma [Maguire *et al* 2009], exhibits greater affinity for the apelin receptor [Hosoya *et al* 2000, Masri *et al* 2006] and is more potent than apelin-36 in competitive inhibition assays [Hosoya *et al* 2000]; however apelin-36 predominates in bovine colostrum [Hosoya *et al* 2000], is less readily displaced from its receptor [Hosoya *et al* 2000; Masri *et al* 2006] and has more sustained biological effects [Hosoya *et al* 2000; Masri *et al* 2006].

Apelin receptors are present on endothelial and vascular smooth muscle cells [Kleinz *et al* 2005] and, in preclinical models, apelin signalling exerts major effects on vascular tone. In *ex vivo* myography studies, apelin causes nitric oxide-dependent vasorelaxation in human mesenteric arteries [Salcedo *et al* 2007] and venoconstriction in endothelium-denuded human saphenous veins [Katugampola *et al* 2001]. In rodent models, exogenous apelin administration causes a rapid fall in

arterial blood pressure [Reaux *et al* 2001; Tatemeoto *et al* 2001; El Messari *et al* 2004; Ishida *et al* 2004; Lee *et al* 2005] mediated by nitric oxide [Tatemoto *et al* 2001] and a concomitant reduction in mean capillary filling pressure [Cheng *et al* 2003] indicating powerful vasodilator and venodilator effects.

As a first step towards characterising the *in vivo* cardiovascular profile of apelin in man, we recently explored the direct vascular actions in man of apelin-36 and (Pyr^{1})apelin-13. Whilst neither isoform induced alterations in peripheral venous tone, both apelin-36 and (Pyr^{1})apelin-13 caused vasodilatation in forearm resistance vessels and the coronary arterial circulation. In this series of studies we sought to characterise and compare peripheral vasodilator responses to arterial administration of two different apelin isoforms within the forearm vascular bed of healthy volunteers and to establish the contribution of the endothelium-derived vasodilators, nitric oxide and prostacyclin, to *in vivo* apelin-mediated vasodilatation.

4.3 METHODS

4.3.1 SUBJECTS

Sixteen healthy volunteers aged between 20 and 24 years participated in these studies, which were performed with the approval of the local Research Ethics Committee, in accordance with the Declaration of Helsinki, and with the written informed consent of all subjects. Participants were not taking any regular medication and had no history of any clinically significant medical condition or symptoms of recent infective or inflammatory illness.

4.3.2 DRUGS

The effects of apelin agonism were assessed using synthetic pharmaceutical grade apelin-36 and (Pyr^{1})apelin-13. Endogenous nitric oxide was inhibited by the use of a nitric oxide clamp, as described previously [Stroes *et al* 1997]. In brief, the nitric oxide synthase inhibitor L-NMMA was continuously infused (8 μmol/min) to achieve maximal inhibition of local nitric oxide synthesis [Vallance *et al* 1989; Calver *et al* 1992; Stroes *et al* 1995]. After 20 minutes of L-NMMA infusion, when steady state blood flow was obtained, SNP an exogenous nitric oxide donor, was co-infused in incremental doses (90-900 ng/min) and titrated to restore baseline FBF. L-NMMA and SNP were then co-infused, at these rates, for the remainder of the study; thus allowing simulation of normal basal nitric oxide activity during continuous inhibition of endogenous nitric oxide synthesis. Dispersible aspirin (600 mg) was dissolved in carbonated water and administered orally 30 minutes before commencement of the study [Heavey *et al* 1985] to inhibit the cyclooxygenase enzyme and thus the production of prostaglandins and thromboxanes.

4.3.3 MEASUREMENTS

All studies were carried out in a quiet, temperature-controlled room (22-25°C). Participants were supine and had abstained from alcohol for 24 hours, and from food and caffeine-containing drinks for at least 4 hours before the study. Heart rate and blood pressure were monitored at regular intervals throughout all studies with a semi-automated, non-invasive oscillometric sphygmomanometer

Subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions. In all studies, saline was infused for the first 20 minutes to allow for equilibration and the intra-arterial infusion rate remained constant at 1 mL/min. Forearm blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [Newby *et al* 1997a; Newby *et al* 1999].

Blood Sampling

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Blood samples (10 mL) were drawn simultaneously from each arm at baseline, during infusion of each dose of apelin and after 42 minutes of 0.9% saline infusion, into tubes containing EDTA and kept on ice before centrifugation at 2000 *g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma apelin concentrations were measured during apelin-36 infusion using a commercially available apelin-12 microplate ELISA Kit.

Effects of apelin-36 and (Pyr^{1})apelin-13 on forearm resistance vessel tone

In Protocol 1 (Figure 4.1), eight subjects attended on two occasions at least one week apart and received continuous incremental doses of either apelin-36 or (Pyr^{1})apelin-13 (0.1-30 nmol/min) for 6 minutes at each dose then, following a 42-minute saline infusion, a continuous dose of 1 nmol/min for 42 minutes. In Protocol 2 (Figure 4.1), the same eight subjects returned for a further two visits at least

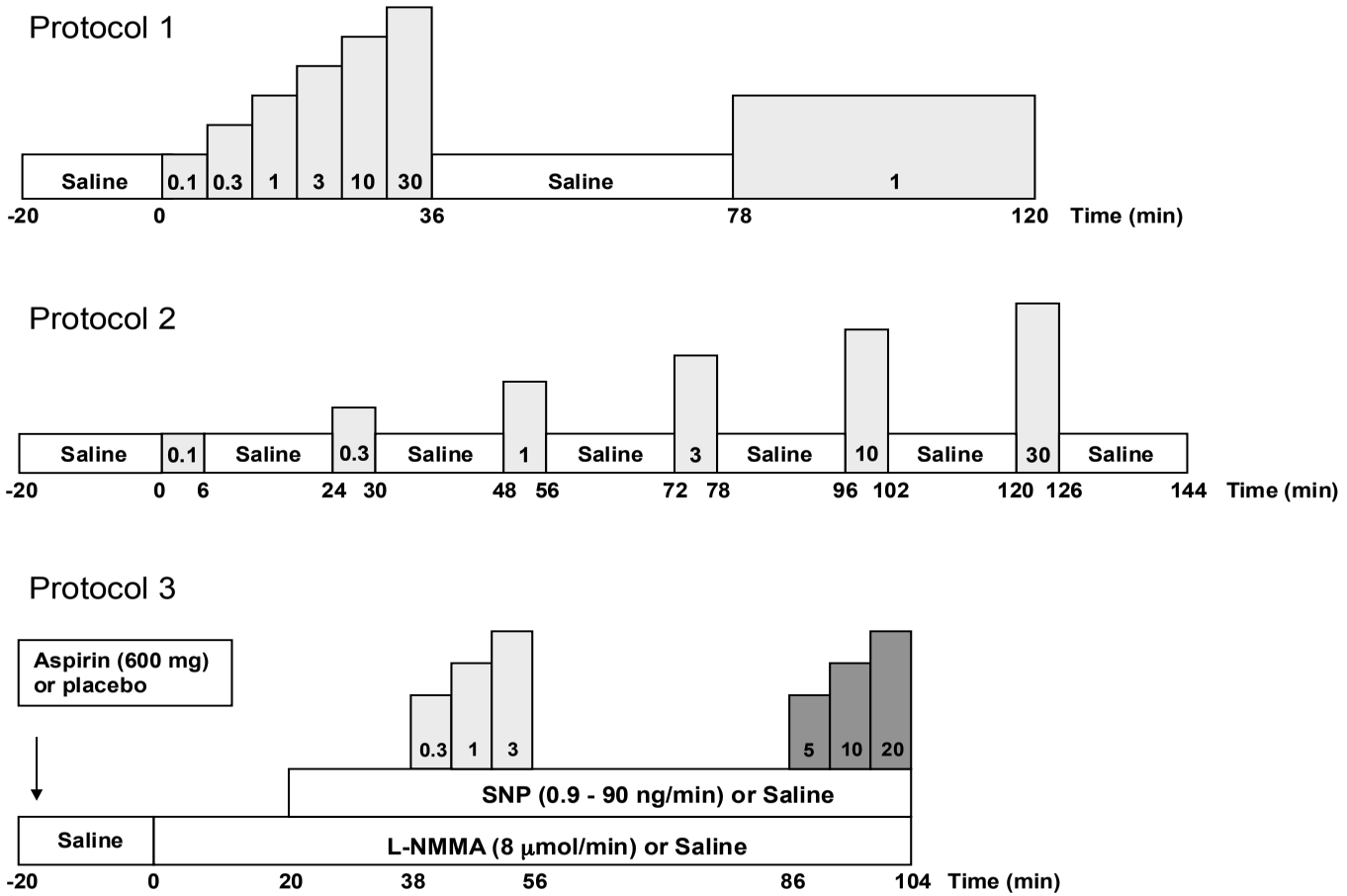

Figure 4.1 Study design. Protocols 1 and 2: Intrabrachial infusions of (Pyr[1])apelin-13 or apelin-36 (■; nmol/min) and saline. Protocol 3: Intrabrachial infusions of (Pyr[1])apelin-13 (■; nmol/min) and acethylcholine (■; μg/min) with co-administration of aspirin or placebo, and 'NO clamp' or saline placebo.
NO - nitric oxide; SNP - sodium nitroprusside; L-NMMA - L-N^{G}-monomethyl-arginine.

one week apart and received discontinuous incremental doses of either apelin-36 or (Pyr^{1})apelin-13 (0.1-30 nmol/min) with each dose separated by an 18-minute saline infusion.

Contribution of nitric oxide and prostacyclin to apelin-mediated vasodilatation

In a 2 x 2 factorial design, eight subjects attended on four occasions at least one week apart and received, in a randomised order: i) the nitric oxide clamp (see above) and oral aspirin, ii) the nitric oxide clamp and oral placebo, iii) saline placebo and oral aspirin, iv) saline placebo and oral placebo (Figure 4.1). Aspirin administration was performed in a double-blinded manner while the nitric oxide clamp was, for practical reasons, performed in a single-blinded manner. On all occasions subjects then received sequential infusions of (Pyr^{1})apelin-13 (0.3, 1, 3 nmol/min) and acetylcholine (5, 10, 20 μg/min) in a double-blinded manner for 6 minutes at each dose with drugs separated by a 30-minute saline infusion. The order of drug infusion was randomised between subjects but kept constant over the four visits for each individual subject.

4.3.4 DATA AND STATISTICAL ANALYSES

Forearm plethysmographic data were analysed in a blinded fashion as described previously [Newby et al 1997a]. The effect of the nitric oxide clamp and aspirin on FBF responses to (Pyr^{1})apelin-13 and acetylcholine were assessed by calculating AUC during drug infusions using the trapezoid method. Apelin assay values were not normally distributed and were therefore log tranformed prior to statistical analysis. Variables are reported as mean ± SEM and analysed using

repeated measures ANOVA with post-hoc Bonferroni corrections and two-tailed Student's *t*-test as appropriate. Statistical significance was taken at the 5% level.

4.4 RESULTS

In all studies, apelin was well tolerated with no significant adverse events. At the highest dose of intra-arterial apelin-36, five subjects experienced a mild patchy non-pruritic localised erythematous swelling in the infused arm that resolved rapidly on cessation of drug infusion. There were no changes in heart rate or blood pressure in any of the studies.

Protocols 1 & 2: Characterisation of apelin-mediated peripheral vasodilatation

(Pyr^1)apelin-13 and apelin-36 increased FBF in the infused arm ($P<0.0001$, ANOVA; Figure 4.2). The response to apelin-36 was dose-dependent ($r^2=0.41$, $P<0.0001$) but there was a rapid plateauing of effect with (Pyr^1)apelin-13 ($r^2=0.003$, $P=0.73$). At the highest dose of 30 nmol/min, infused FBF was greater during apelin-36 infusion compared with (Pyr^1)apelin-13 infusion (Figure 4.3A; $P<0.05$).

Where doses were separated by saline washouts (Figure 4.4), blood flow declined over 18 minutes after each dose, but did not return to baseline. Following continuous incremental dose infusion, FBF declined gradually over 42 minutes with both isoforms (Figure 4.2; $P<0.0001$, ANOVA). With (Pyr^1)apelin-13, FBF remained elevated at 6 minutes during saline washout ($P<0.05$) but not thereafter. However

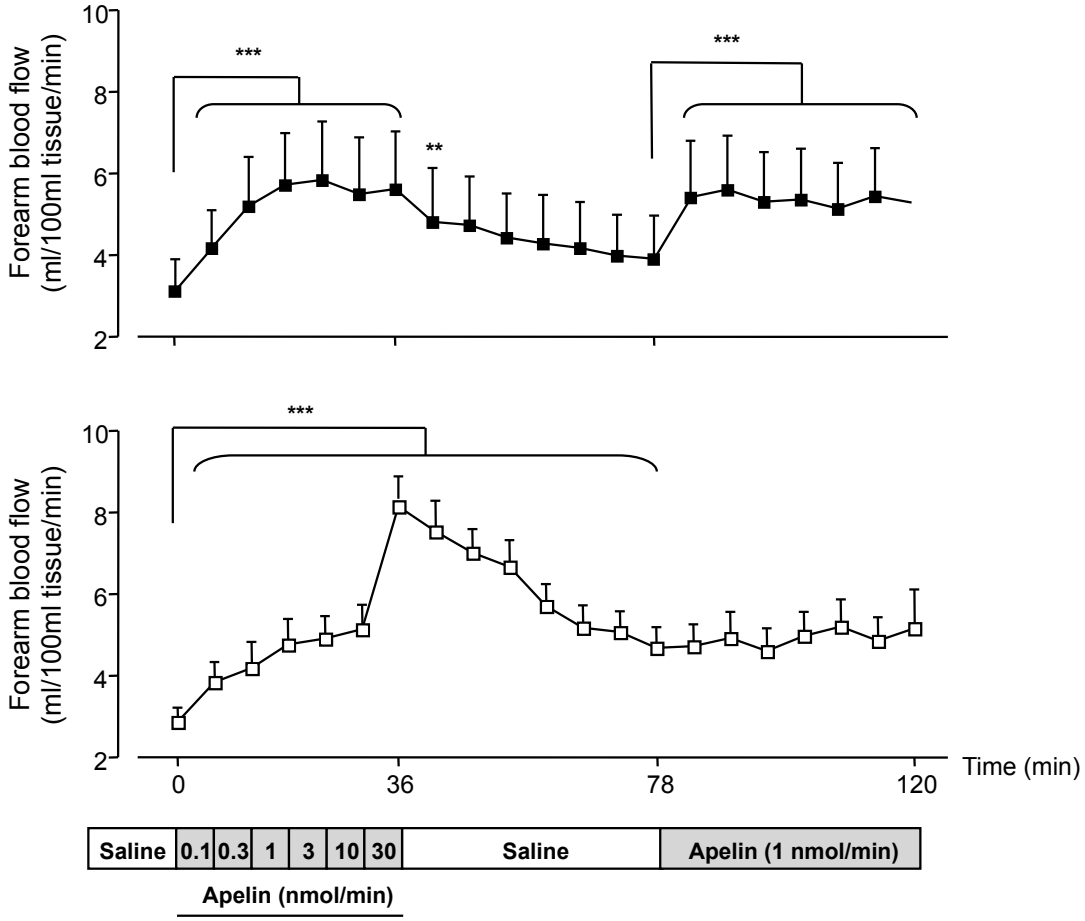

Figure 4.2 Infused FBF in Protocol 1 during infusion of (Pyr[1])apelin-13 (■) and apelin-36 (□). One-way ANOVA with repeated measures. **P<0.01, ***P<0.001, post-hoc Bonferroni tests *versus* baseline. FBF - forearm blood flow; ANOVA - analysis of variance.

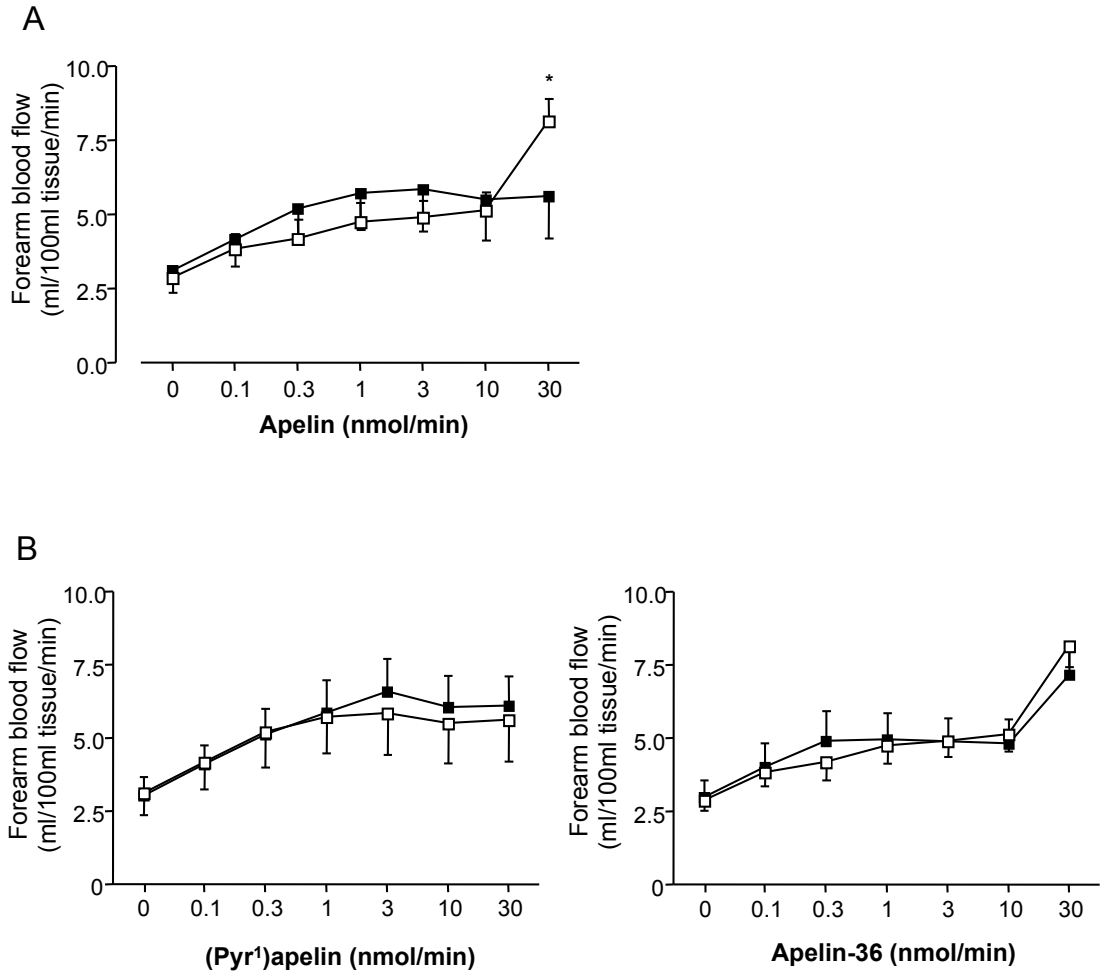

Figure 4.3 Infused FBF A: during incremental infusion of (Pyr[1])apelin-13 (■) versus apelin-36 (□) in Protocol 1; 2-way ANOVA with repeated measures. *P<0.05, post-hoc bonferroni tests. B: during incremental infusion of (Pyr[1])apelin-13 and apelin-36 in the presence (■) and absence (□) of saline washouts (for both: P>0.05, 2-way ANOVA with repeated measures).
FBF - forearm blood flow; ANOVA - analysis of variance.

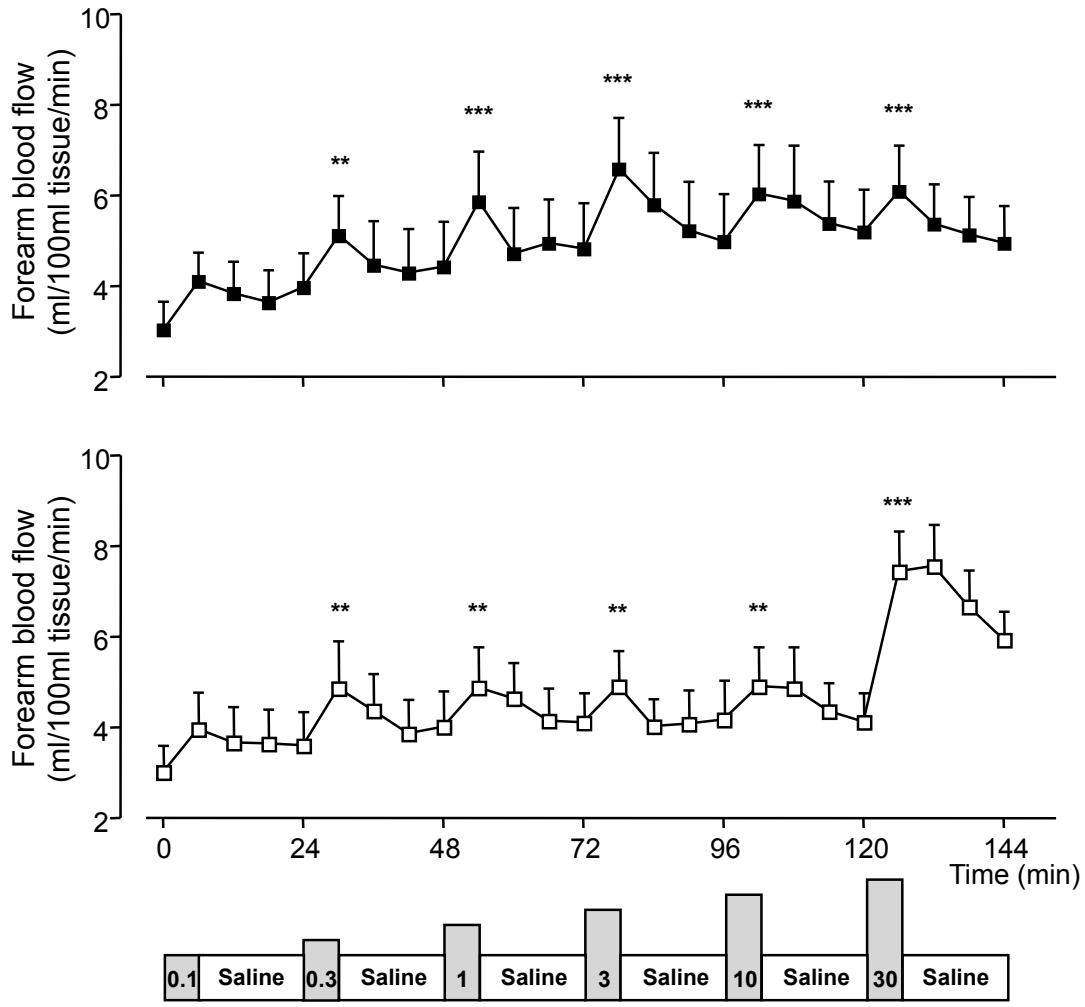

Figure 4.4 Infused FBF in Protocol 2 during infusion of (Pyr[1])apelin-13 (■) and apelin-36 (□). One-way ANOVA with repeated measures. **P<0.01, ***P<0.001, post-hoc Bonferroni tests *versus* baseline. FBF - forearm blood flow; ANOVA - analysis of variance.

with apelin-36, infused FBF remained elevated throughout the washout period ($P<0.0001$).

Forty-two minutes after incremental dose infusion of (Pyr^1)apelin-13, a further continuous infusion of (Pyr^1)apelin-13 at 1 nmol/min elicited a sustained increase in infused FBF ($P<0.0001$). As noted above, infused FBF failed to return to baseline following incremental apelin-36 infusion. Subsequently, infused FBF failed to rise further during continuous infusion of apelin-36 at 1 nmol/min but remained elevated with respect to baseline ($P<0.001$). The inclusion of 18-minute saline washouts between dose escalations (Figure 4.3B) did not affect vasodilatation to either (Pyr^1)apelin-13 ($P=0.68$) or apelin-36 ($P=0.93$).

With both apelin isoforms, FBF increased in the non-infused arm ($P<0.0001$, ANOVA) at 30 nmol/min ($P<0.05$, post-hoc Bonferroni tests; data not shown). Plasma apelin concentrations in the infused arm rose with increasing doses of apelin-36 (Figure 4.5; $P<0.0001$) and increased in the non-infused arm from 10 nmol/min ($P<0.01$).

Protocol 3: Contribution of nitric oxide and prostacyclin to apelin-mediated vasodilatation

L-NMMA reduced blood flow in the infused arm (3.48 ± 0.41 - 2.10 ± 0.17 mL/100 mL/min, $P<0.001$) but this was restored with SNP co-infusion (3.20 ± 0.33 mL/100 mL/min, $P>0.05$ *versus* baseline). Both (Pyr^1)apelin-13

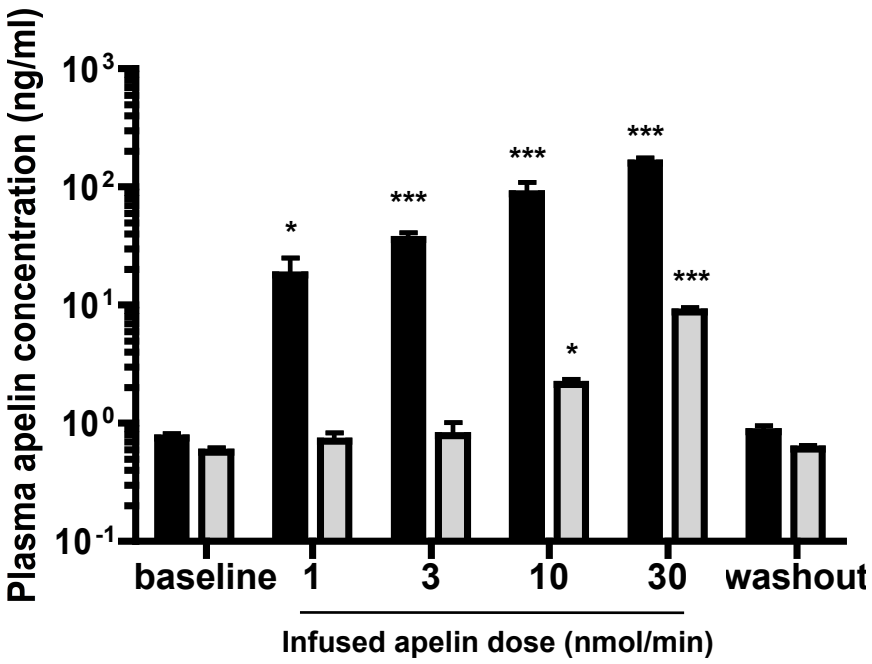

Figure 4.5 Plasma apelin concentration in venous blood samples from infused (■) and non-infused arm (■) during intrabrachial apelin-36 infusion. *P<0.05, ***P<0.001, one-way ANOVA with post-hoc Bonferroni tests.
ANOVA - analysis of variance.

(P<0.001) and acetylcholine (P<0.001) increased blood flow in the infused arm (Figure 4.6). Co-infusion of the nitric oxide clamp inhibited the response to (Pyr^{1})apelin-13 and acetylcholine but aspirin had no effect in the presence or absence of the nitric oxide clamp (Figure 4.6).

4.5 DISCUSSION

We have assessed the direct *in vivo* effects of the two predominant apelin isoforms in man in the human forearm vascular bed. Apelin causes rapid onset, sustained and reproducible vasodilatation in peripheral resistance vessels through a nitric oxide-dependent mechanism.

4.5.1 CHARACTERISATION OF VASODILATOR RESPONSES TO APELIN

We measured changes in FBF and plasma apelin concentrations during local intra-arterial infusions of two apelin isoforms: apelin-36, the full-length mature peptide, and (Pyr^{1})apelin-13, a shorter C-terminal fragment. At infusion rates >3 nmol/min, we detected a rise in plasma apelin concentrations in the non-infused arm indicating spillover of infused apelin into the systemic circulation. This spillover limits interpretation of blood flow changes at the higher doses of apelin because of potential concomitant effects on myocardial contractility and activation of neurohumoral reflexes. However, at apelin doses ≤3 nmol/min, changes in blood flow can be solely ascribed to direct local vascular effects.

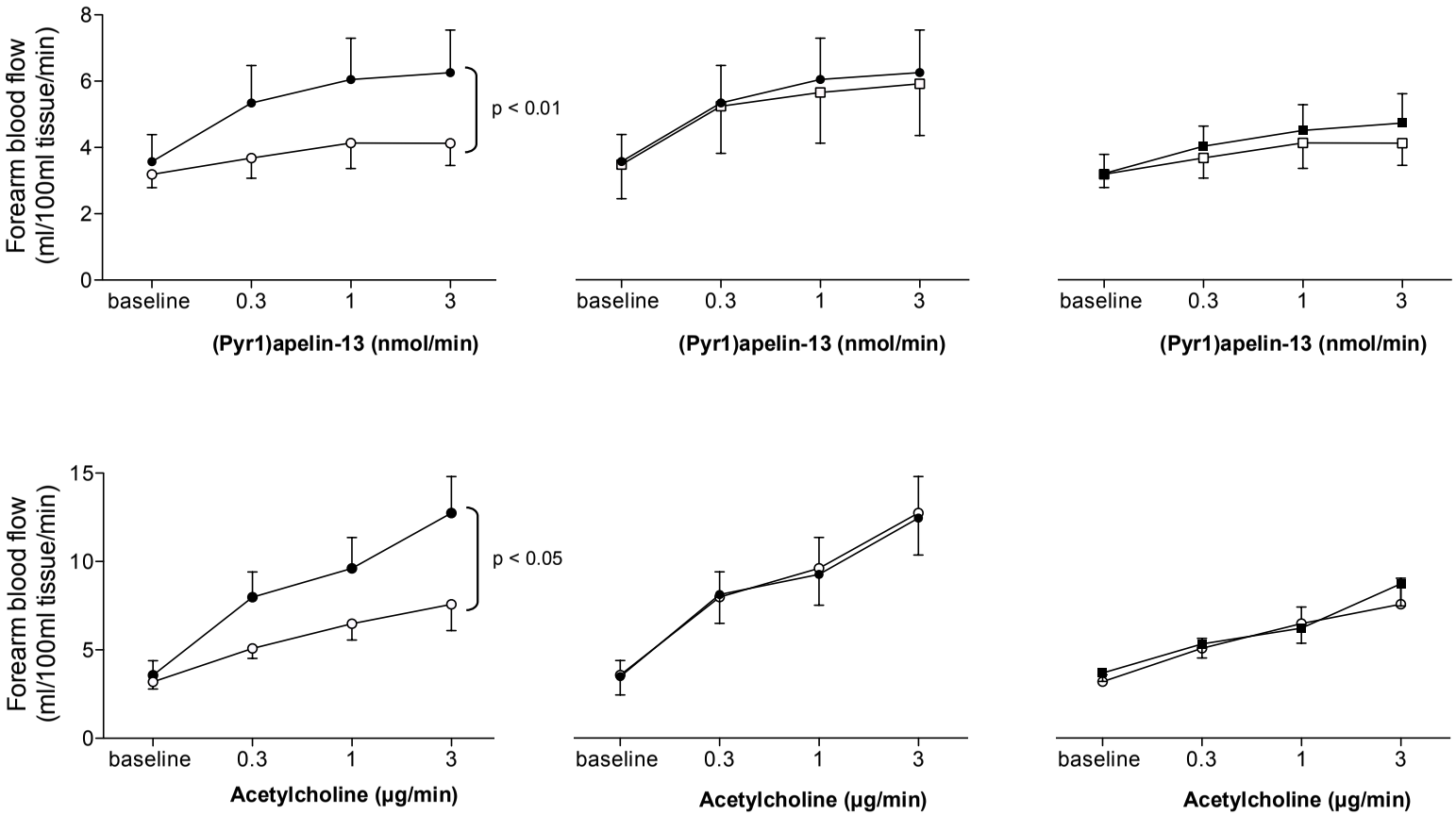

Figure 4.6 Infused FBF in subjects during infusion of (Pyr[1])apelin-13 (top) or acetylcholine (bottom) in the presence (○) or absence (●) of the NO clamp (left hand panel), in the presence (□) or absence (●) of aspirin without the NO clamp (middle panel) and in the presence (■) or absence (○) of aspirin with co-administration of the NO clamp (right hand panel). For dose-responses to (Pyr[1])apelin-13 and acetylcholine, P<0.001 for all (one-way ANOVA). For placebo *versus* NO clamp (AUC): (Pyr[1])apelin-13 (P<0.01); acetylcholine (P<0.05). For placebo *versus* aspirin without NO clamp (AUC): (Pyr[1])apelin-13 (P=0.7); acetylcholine (P=0.9). For placebo *versus* aspirin with NO clamp (AUC): (Pyr[1])apelin-13 (P=0.6); acetylcholine (P=0.7).
FBF - forearm blood flow; NO - nitric oxide; ANOVA - analysis of variance; AUC - area under the curve.

The onset of vasodilatation in these studies was rapid, occurring within 6 minutes. However, the offset was slow, with blood flow in the infused arm consistently remaining above baseline during washout periods and persisting for up to 42 minutes. This prolonged offset of action is unusual but has been described with other agonists, such as the vasopressin V2 receptor agonist, desmopressin [Affolter *et al* 2003]. It is consistent with *in vitro* data showing a prolonged response to apelin stimulation in various microphysiometric assays, particularly with apelin-36 [Masri *et al* 2006]. The prolonged offset cannot be explained by persistence of the peptide in plasma because vasodilatation persisted after plasma apelin concentrations had returned to baseline. Indeed, the results of these studies suggest apelin has a relatively short plasma half-life of no longer than 8 minutes.

In comparison with other established endothelium-dependent vasodilators, such as acetylcholine [Newby *et al* 1997b], bradykinin [Mills *et al* 2005] and substance P [Newby *et al* 1997c], the vasodilatation induced by apelin was relatively modest with a maximum increase in FBF of 70-80%. With both apelin isoforms, vasodilatation appeared to plateau at doses >0.1 nmol/min. One potential explanation for this effect is desensitisation of the apelin receptor to ligand activation. *In vitro*, stimulation with either (Pyr^{1})apelin-13 or apelin-36 causes rapid and dose-dependent internalisation of apelin receptors [Reaux *et al* 2001; Zhou *et al* 2003] and several apelin-mediated signalling cascades are rapidly attenuated by repeated ligand exposure [Choe *et al* 2000; Masri *et al* 2006]. However, in our studies, continuous infusion of (Pyr^{1})apelin-13 elicited sustained, near maximal vasodilatation over a 42-minute period. In addition, we did not find a greater vasodilator response with incremental

discontinuous infusions than with incremental continuous doses. We therefore believe it more likely that the observed plateau in blood flow represents a true ceiling of the vasodilator response to apelin rather than desensitisation.

4.5.2 COMPARISON OF APELIN ISOFORMS

Although shorter apelin fragments are reported to have more potent depressor activity than apelin-36 in rodents [Tatmemoto *et al* 2001] vasodilator responses to the two isoforms in this study did not differ at locally active doses. At the maximum dose of 30 nmol/min, blood flow in the infused arm increased sharply with apelin-36 and was greater than that observed with (Pyr^{1})apelin-13. However this coincided with an increase in systemic plasma apelin concentration and blood flow in the non-infused arm. Given the apparent plateauing of blood flow responses to apelin-36 at subsystemic doses and the known inotropic effects of apelin in preclinical models [Szokodi *et al* 2002; Ashley *et al* 2005], it is tempting to speculate that this may have reflected an increase in cardiac contractility. On the other hand, we saw no concomitant effect on heart rate or blood pressure and the purpose of the present study was to assess the direct vascular effects of apelin. Further appropriately designed studies, with higher systemic doses, are needed to address the question of whether intravenous apelin infusion can affect systemic haemodynamic and cardiac variables.

Whilst both isoforms elicited a rapid onset of vasodilatation, the offset was more prolonged following apelin-36 infusion. Indeed the duration of the vasodilatator response to apelin-36 remains uncertain as it exceeded the washout time employed in

these studies but it is at least 42 minutes. Recent *in vitro* work exploring the interaction of different apelin isoforms with the apelin receptor may provide insights into the differing offset of response. Whilst (Pyr^{1})apelin-13 has a greater binding affinity for the apelin receptor, apelin-36 exhibits a much slower dissociation, leading to prolonged receptor activation [Masri *et al* 2006]. Cell lines expressing the apelin receptor that are pretreated with apelin-36 show no clear response to subsequent apelin stimulation because intracellular responses e.g. inhibition of adenylate cyclase, remain maximally activated even after 2 hours [Masri *et al* 2006]. In contrast, intracellular responses to apelin-13 are more transient and show a further increase on re-exposure [Masri et al 2006]. Thus it appears that the slow dissociation of apelin-36 effectively locks the receptor in an active configuration. Such pharmacodynamic differences might potentially underpin distinct physiological roles for the different apelin isoforms *in vivo*.

4.5.3 CONTRIBUTION OF NITRIC OXIDE AND PROSTACYCLIN

To investigate the mechanism of apelin-mediated vasodilatation, we examined the effect of local inhibition of nitric oxide synthesis and systemic inhibition of prostaglandin synthesis, alone and in combination, on blood flow responses to subsystemic doses of (Pyr^{1})apelin-13. We found that vasodilatation to (Pyr^{1})apelin-13 was attenuated by two-thirds during the nitric oxide clamp compared with saline placebo, indicating that vasodilatation to apelin, is mediated predominantly by endothelial nitric oxide generation. As expected, the nitric oxide clamp also inhibited acetylcholine-mediated vasodilatation, which is known to be mediated in part by nitric oxide [Calver *et al* 1992]. In contrast to local inhibition of

nitric oxide synthase, systemic inhibition of prostanoid generation with oral aspirin did not alter vasodilatation to either (Pyr^{1})apelin-13 or acetylcholine, alone or in combination with the nitric oxide clamp. The dose of aspirin used in this study has previously been shown to inhibit bradykinin-stimulated endothelial production of prostacylin by at least 85% [Heavey *et al* 1985], suggesting that prostanoids do not provide a major contribution to apelin-mediated vasodilatation. The failure of aspirin to inhibit acetylcholine-mediated vasodilatation in the human forearm is consistent with previous reports [Noon *et al* 1998; Campia *et al* 2002]. Our findings *in vivo* are in close agreement with Salcedo *et al* who recently demonstrated that nitric oxide synthase but not cyclooxygenase inhibition attenuated relaxation to apelin in human mesenteric arteries *in vitro* [Salcedo *et al* 2007].

In the absence of functioning endothelium apelin induces vasoconstriction *ex vivo* by a direct action on vascular smooth muscle cells [Katugampola *et al* 2001]. However, in these studies we observed no evidence of apelin-mediated vasoconstriction, even during combined inhibition of nitric oxide and prostacyclin synthesis.

4.5.4 CONCLUSIONS

Apelin-mediated vasodilatation in the human forearm circulation is reproducible, sustained and mediated predominantly by nitric oxide. The vasodilator responses to apelin-36 and (Pyr^{1})apelin-13 are equivalent in magnitude but more prolonged with apelin-36.

CHAPTER 5

CARDIAC AND SYSTEMIC HAEMODYNAMIC EFFECTS OF APELIN IN MAN

Japp AG, Cruden NL, Barnes G *et al.*
Acute cardiovascular effects of apelin in humans:
potential role in patients with chronic heart failure.
Circulation 2010;**121**:1818-1827.

5.1 SUMMARY

Apelin has major cardiovascular effects in preclinical models. The objectives of this study were to establish the effects of acute apelin administration on cardiac and systemic haemodynamic variables in man. Systemic haemodynamic variables were measured non-invasively by TEB during randomised doubled-blind intravenous infusions of apelin-36 or (Pyr^{1})apelin-13 (30-300 nmol/min) and matched saline placebo in eight healthy volunteers. Left ventricular pressures were measured invasively by pressure wire in six patients undergoing diagnostic coronary angiography following intracoronary bolus injections of apelin-36 (20 and 200 nmol in 2 mL) and 0.9% saline (2 x 2 mL) in a double-blind randomised manner, and a single-blinded non-randomised bolus of GTN (100 μg in 2 mL). Intracoronary apelin-36 increased dP/dtmax, and reduced peak and end-diastolic left ventricular pressures (all $P<0.05$). Systemic infusions of (Pyr^{1})apelin-13 and apelin-36 (30-300 nmol/min) increased cardiac index and heart rate and lowered peripheral vascular resistance (all $P<0.01$). Acute apelin administration in man increases cardiac contractility and cardiac output whilst lowering peripheral vascular resistance. This profile of cardiovascular actions suggests therapeutic potential for apelin receptor agonism in the treatment of heart failure.

5.2 INTRODUCTION

The apelin receptor (previously known as 'APJ') is a novel G protein-coupled receptor, originally discovered in 1993 [O'Dowd *et al* 1993] and subsequently paired with its endogenous ligand, apelin in 1998 [Tatemoto *et al* 1998]. Apelin receptors are present on endothelial cells, vascular smooth muscle cells and cardiomyocytes. [Kleinz *et al* 2005] and, in preclinical models, apelin signalling exerts major effects on both vascular tone and cardiac contractility. In isolated rat hearts, apelin is the most potent endogenous inotrope yet described [Szokodi *et al* 2002] and, in *ex vivo* myography studies, it causes vasorelaxation in human mesenteric artery that is attenuated by inhibition of nitric oxide but not prostacyclin [Salcedo *et al* 2007]. In rodents, apelin increases cardiac contractility *in vivo* [Ashley *et al* 2005; Atluri *et al* 2007] and causes a rapid fall in both arterial blood pressure and systemic venous tone [Tatemoto *et al* 2001; Cheng *et al* 2003; Lee *et al* 2005] with corresponding reductions in left ventricular afterload and preload [Ashley *et al* 2005]. If replicated in clinical studies such a profile of cardiovascular actions might suggest a potential role for apelin receptor agonism in the treatment of heart failure.

We recently provided the first evidence that apelin has vasoactive actions *in vivo* in man [Japp *et al* 2008]. We demonstrated that local apelin administration causes vasodilatation in the coronary circulation and peripheral resistance vessels with no apparent effect on peripheral venous tone. In the current series of studies, we sought to further characterise the *in vivo* cardiovascular profile of apelin in man by establishing its effects on cardiac and systemic haemodynamic variables.

5.3 METHODS

All studies were performed with the written informed consent of volunteers, the approval of the local Research Ethics Committee and in accordance with the Declaration of Helsinki.

5.3.1 SUBJECTS

Eight healthy volunteers aged between 21 and 27 years and six patients attending for elective outpatient diagnostic coronary angiography participated in these studies. Healthy volunteers were not taking any regular medication and had no history of any clinically significant medical condition or symptoms of recent infective or inflammatory illness. Patients attending for diagnostic angiography were excluded if they were found to have left main stem stenosis or severe coronary artery stenosis, previous coronary intervention, and clinical or echocardiographic evidence of cardiac failure. Patients withheld their usual medications on the day of the study until completion of the study protocol and all participants abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least 4 hours before each study.

5.3.2 DRUGS

The effects of apelin receptor agonism were assessed using synthetic pharmaceutical grade apelin-36 and (Pyr^{1})apelin-13. GTN was administered as a control vasodilator.

5.3.3 MEASUREMENTS

All studies were carried out in a quiet, temperature-controlled room (23-25°C) with subjects in the supine position.

Myocardial Contractility Studies

Following diagnostic coronary angiography, a coronary guide catheter was engaged in the ostium of the left coronary artery. Coronary blood flow was measured as described in Chapter 3. A further catheter was inserted into the left ventricle and a 0.014-inch pressure wire placed in the left ventricular cavity to allow continuous measurement of left ventricular pressure [Duckett *et al* 2011; Ginks *et al* 2011]. The output from the pressure wire was connected to a RadiAnalyzer® and transferred to a laptop computer with PhysioMon® software to give curves showing real-time blood pressure and left ventricular dP/dtmax. Ten-second samples of pressure recordings within the left ventricle were taken before and at 60 seconds after each drug bolus and used to calculate dP/dtmax, LV max and LVEDP.

Systemic Haemodynamic Studies

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms to allow drug infusion and sampling of venous blood. Blood pressure and heart rate were recorded with a semi-automated non-invasive oscillometric sphygmomanometer; mean arterial pressure was defined as the sum of the diastolic blood pressure and a third of the pulse pressure. Cardiac output was measured non-invasively using a TEB monitor [Northridge *et al* 1990] and corrected

for body surface area to give cardiac index. At each time point, cardiac output was taken as the mean of three recordings, each recording representing the average of 16 consecutive heartbeats. Peripheral vascular resistance index was calculated as mean arterial pressure divided by cardiac index and expressed in dyne.s/cm^5/m^2. Continuous electrocardiographic monitoring was performed during all studies.

Apelin Assay

Blood samples (10 mL) were drawn into tubes containing EDTA and kept on ice before centrifugation at 2000 *g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma apelin-36 concentration was measured using a commercially available apelin-12 micro-plate ELISA Kit.

5.3.4 PROTOCOL DESIGN

Protocol 1: Myocardial contractility studies

As previously described in Chapter 3, six subjects received intracoronary boluses via the coronary guide catheter of apelin-36 (20 and 200 nmol in 2 mL) and 0.9% saline (2 x 2 mL) in a double-blind randomised manner, followed by a single-blinded non-randomised bolus of GTN **(**100 μg in 2 mL). All drug boluses were followed by a 2 mL 0.9% saline flush and 5-minute washout intervals were allowed between drugs. Left ventricular dP/dtmax, LV max and LVEDP, as well as coronary blood flow (see Chapter 3) were calculated at baseline and before and after each drug bolus.

Protocol 2: Systemic haemodynamic studies

Eight subjects attended on two occasions at least one week apart. In a double-blind randomised manner, three incremental intravenous doses of either apelin-36 or (Pyr^{1})apelin-13 (30, 100, 300 nmol/min) were given for 5 minutes at each dose and compared with three matched saline placebo infusions. Doses of apelin were determined from our previous study [Japp *et al* 2008] where 30 nmol/min caused a rise in systemic plasma apelin concentrations. Apelin and saline infusions were separated by a 30-minute saline infusion. Haemodynamic recordings were made at 10-minute intervals prior to commencement of drug infusion and at 5-minute intervals thereafter. Venous blood samples were obtained at baseline, at peak drug infusion and at 5 and 30-minute post drug infusion for measurement of plasma apelin concentration.

5.3.5 DATA AND STATISTICAL ANALYSES

Cardiac and systemic haemodynamic variables were analysed in a blinded fashion. Variables are reported as mean ± SEM and analysed using repeated measures ANOVA with post-hoc Bonferroni corrections and two-tailed Student's *t*-test as appropriate. Statistical significance was taken at the 5% level.

5.4 RESULTS

All studies were well tolerated with no serious adverse events or ECG changes during apelin administration.

5.4.1 MYOCARDIAL CONTRACTILITY

Patients were aged 60 ± 4 years and five were male. All patients had near normal coronary arteries with no haemodynamically significant flow limiting stenoses (<25% luminal stenosis). Owing to a technical computer failure during one of the studies, incomplete haemodynamic data were obtained for one subject. Consequently the analyses for left ventricular pressure are based on n=5.

As described in Chapter 3, both the higher dose of apelin and GTN caused an increase in coronary blood flow. There were no significant changes in haemodynamic variables following injection of 20 nmol apelin-36 (data not shown). In comparison with placebo, both 200 nmol apelin-36 and GTN caused an increase in dP/dtmax and a slight reduction in LV max and LVEDP (Figure 5.1). In comparison with placebo, GTN caused a fall in mean arterial pressure (94 ± 8 *versus* 80 ± 9 mmHg, P<0.01) and rise in heart rate (67 ± 5 *versus* 69 ± 5 bpm, P<0.001) but apelin had no effect on blood pressure (94 ± 8 *versus* 94 ± 7 mmHg, P=0.9) or heart rate (67 ± 5 *versus* 68 ± 5 bpm, P=0.3).

5.4.2 SYSTEMIC HAEMODYNAMICS

Saline placebo infusion had no effect on any of the haemodynamic variables (P>0.05, one-way ANOVA). Compared with saline placebo, both (Pyr^{1})apelin-13 and apelin-36 increased heart rate and cardiac output whilst reducing peripheral vascular resistance (Figures 5.2 and 5.3). There appeared to be no clear dose-response relationship at the doses employed in the present study and, consistent with our previous study [Japp *et al* 2008], apelin-36 had a more sustained offset of action

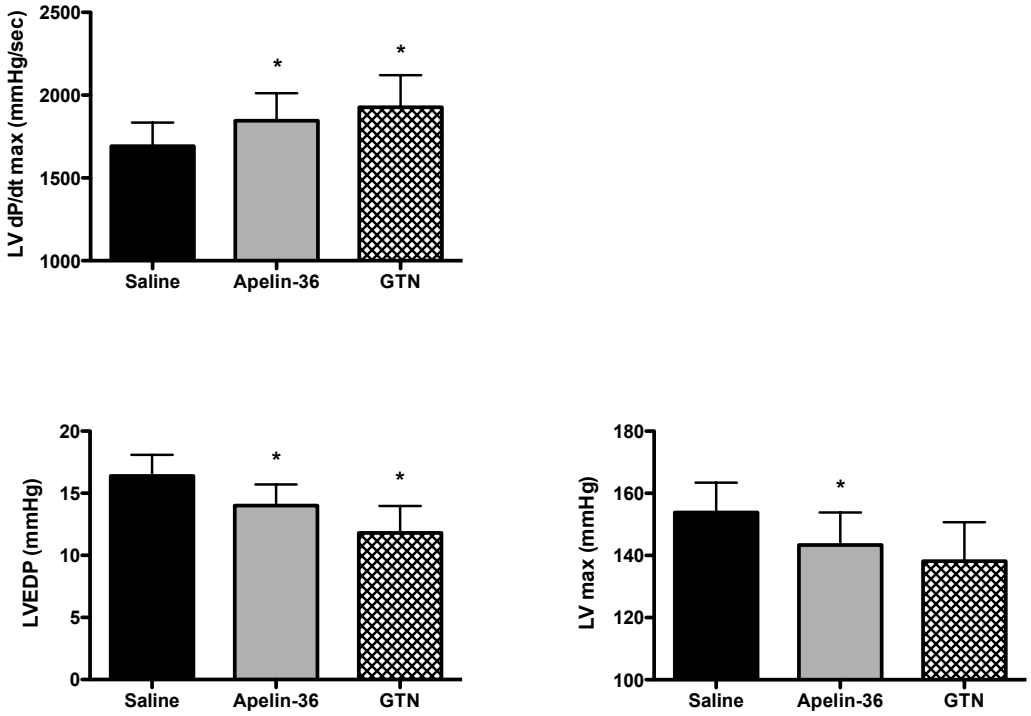

Figure 5.1 Left ventricular pressure variables following bolus injection of apelin-36 (200 nmol), GTN (100 μg) and 0.9% saline. *P<0.05, paired student's *t*-test *versus* 0.9% saline.
GTN - glyceryl trinitrate; LV dP/dtmax - left ventricular maximum rate of rise in pressure; LVEDP - left ventricular end-diastolic pressure; LV max - maximum left ventricular systolic pressure.

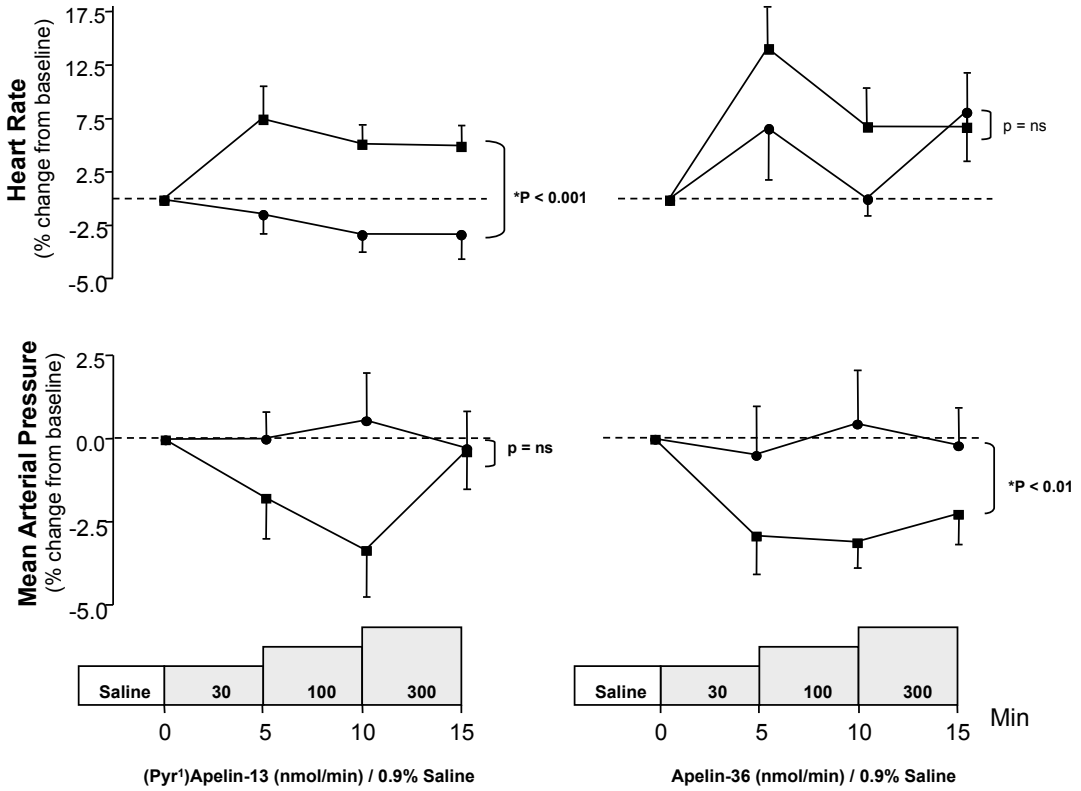

Figure 5.2 Heart rate and blood pressure during infusion of apelin (■) or matched saline placebo (●) in healthy subjects. Two-way ANOVA (apelin *versus* placebo).
ANOVA - analysis of variance; ns - not significant.

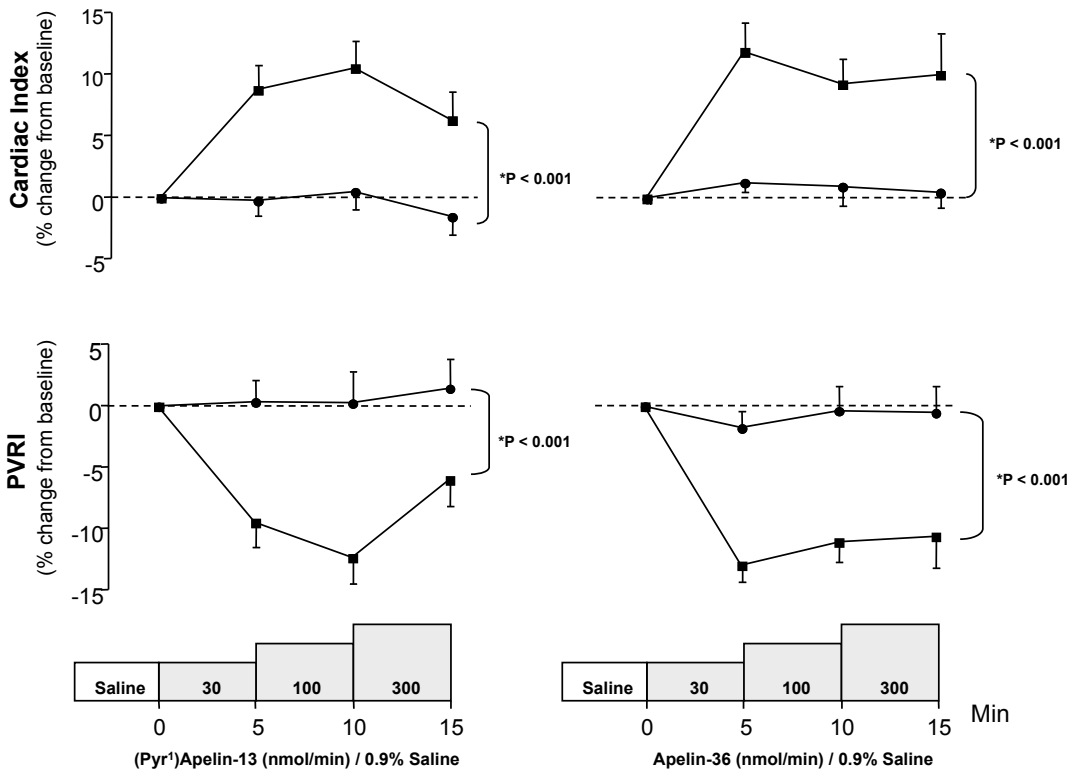

Figure 5.3 Cardiac index and peripheral vascular resistance index during infusion of apelin (■) or matched saline placebo (●) in healthy subjects. Two-way ANOVA (apelin *versus* placebo).
ANOVA - analysis of variance; PVRI - peripheral vascular resistance index.

than (Pyr^{1})apelin-13 (Figure 5.4). The small rise in heart rate was seen early and was not sustained (Figure 5.4). With both isoforms there was a trend towards a reduction in blood pressure that did not reach statistical significance (Figure 5.3). Plasma concentration of apelin-36 increased from 0.24 ± 0.07 pg/mL at baseline to 115.86 ± 16.97 pg/mL during maximal infusion of apelin-36 ($P<0.0001$); and by 5 minutes post infusion had fallen to 39.95 ± 7.82 pg/mL ($P=0.002$ *versus* peak infusion) suggesting a plasma half-life of less than 5 minutes. By 30 minutes post infusion, plasma apelin concentrations had returned to near baseline (1.56 ± 0.55) and did not differ from baseline values ($P>0.05$).

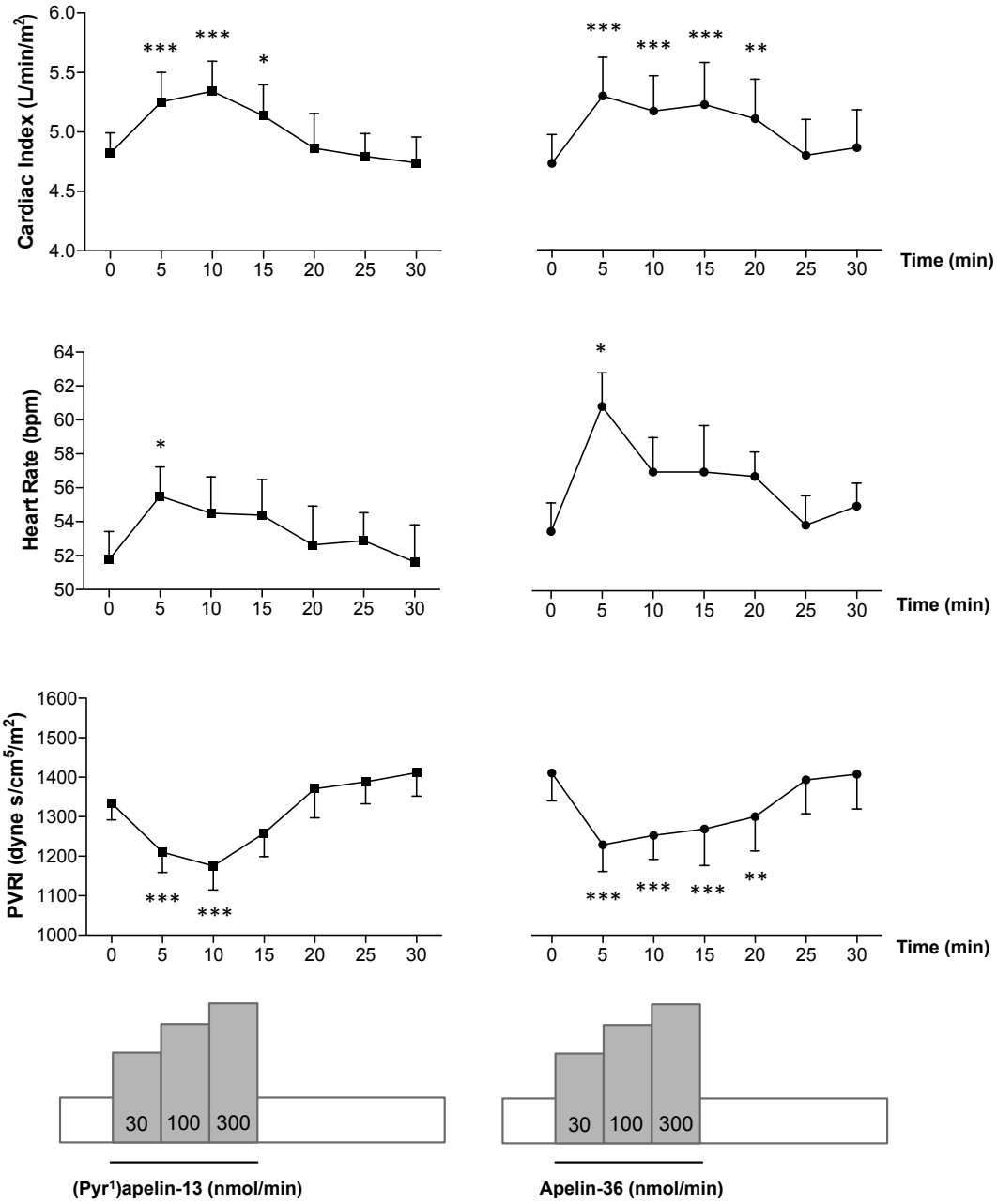

Figure 5.4 Systemic haemodynamic variables during graded infusions of (Pyr[1])apelin-13 (■) or apelin-36 (●) followed by 0.9% saline washout. *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA with post-hoc Bonferroni tests (apelin *versus* baseline).
ANOVA - analysis of variance; PVRI - peripheral vascular resistance index.

5.5 DISCUSSION

For the first time, we have demonstrated that acute apelin infusion in man reduces peripheral arterial resistance, alters cardiac loading conditions and increases cardiac contractility and output.

In keeping with its actions in preclinical models, acute apelin infusion in man decreases peripheral vascular resistance whilst increasing heart rate and cardiac output. We have previously shown that local apelin infusion causes nitric oxide-mediated vasodilatation in the human forearm circulation [Japp *et al* 2008]. In the present study we extend this work by confirming that the direct vascular actions of apelin modulate systemic haemodynamics. The increase in heart rate in our study occurred early and was not sustained. In rodents a similar rise in heart rate following apelin administration is abolished by ganglionic blockade [Cheng *et al* 2003]. We therefore believe it is more likely to represent a reflex sympathetic response to peripheral vasodilatation than a direct chronotropic effect. Similarly, the observed increase in cardiac output is likely to be explained at least in part through peripheral arterial vasodilation via a reduction in afterload and activation of compensatory neurohormonal reflexes. In rodents apelin-mediated vasodilatation is associated with a marked depressor response [Tatemoto *et al* 2001; Lee *et al* 2005] whereas in our study no significant blood pressure changes were observed. Of note the blood pressure lowering effects of apelin in preclinical models peak within 60 seconds and decline rapidly thereafter [Lee *et al* 2005]. However we measured blood pressure at 5-minute intervals during apelin infusion. Additionally,

our study may have been underpowered to detect a small reduction in blood pressure. Consequently, this apparent discrepancy may reflect an inability of our protocol to detect an early transient rise in blood pressure rather than a true interspecies difference.

We assessed the haemodynamic effects of two apelin isoforms: apelin-36, the full-length mature peptide, and (Pyr^{1})apelin-13, a shorter C-terminal fragment. In preclinical studies the shorter isoform is reported to exhibit greater binding affinity [Hosoya *et al* 2000; Masri *et al* 2006] and more potent cardiovascular effects [Tatemoto *et al* 2001]. Here the magnitude of haemodynamic effects elicited by the two isoforms was equivalent, though, consistent with *in vitro* data and our findings in the forearm circulation, the responses to apelin-36 exhibited a slower offset. The changes in systemic haemodynamic variables did not demonstrate a clear dose-response relationship and appeared to demonstrate a flat dose-response. This was also seen in our previous studies in the peripheral forearm circulation [Japp *et al* 2008] and may reflect our selection of doses or an 'all-or-nothing' response.

The observed rise in cardiac output during systemic apelin administration may, at least in part, have been a reflex response to peripheral vasodilatation. However preclinical data suggest that apelin also exerts direct positive inotropic effects. [Szokodi *et al* 2002]. In order to assess the *in vivo* cardiac effects of apelin in man we measured left ventricular pressures during intracoronary apelin administration. Consistent with direct effects on myocardial contractility, apelin caused an increase in dP/dtmax. However the increase occurred only with the higher dose of apelin and

was associated with a concomitant reduction in both LV max and LVEDP indicating likely systemic vascular effects. Whilst we did not measure peripheral plasma apelin concentrations during these studies, the administered dose exceeded that required to generate systemic effects and a rise in plasma apelin levels in our intravenous studies. Of note, a very similar haemodynamic profile of effects occurred following GTN injection. We therefore believe it is likely that there was overspill of both apelin and GTN in to the peripheral circulation leading to alterations in peripheral vascular tone. This interpretation is supported by the apparent absence of any direct cardiac effects following injection of the lower dose of apelin and would be in keeping with the clear evidence of peripheral arterial vasodilatation in our forearm and systemic infusion studies. Consequently, our data do not provide definitive evidence of a direct effect of apelin on myocardial contractility *in vivo* in man. However, in contrast to GTN, which caused a marked fall in systemic blood pressure and corresponding rise in heart rate, the higher dose of apelin had no effect on blood pressure or heart rate, raising the possibility of an additional direct inotropic effect.

Although the presumed systemic spillover during intracoronary administration confounds assessment of the direct cardiac effects of apelin, the resultant changes in loading conditions provide the first evidence that the direct vascular effects of apelin couple to the left ventricle. Furthermore, the observed reduction in LVEDP suggests for the first time that apelin causes venodilatation in man. Whilst we have previously reported that apelin has no apparent effect on venous tone in superficial hand veins [Japp *et al* 2008] these veins are not representative of other venous beds and are of less haemodynamic importance than deep capacitance veins [Schmitt *et al* 2002].

The finding that apelin reduces interspecies LVEDP in man is consistent with evidence of a powerful venodilator effect in rodents [Cheng *et al* 2003].

Reductions in myocardial expression of apelin and the apelin receptor have been demonstrated in experimental rodent models of heart failure [Iwanaga *et al* 2006; Jia *et al* 2006]. Failing human hearts exhibit similarly altered expression patterns and patients [Foldes *et al* 2003], with severe chronic heart failure have reduced plasma apelin concentrations [Chong *et al* 2006]. Given the cardiovascular actions of apelin described above, this apparent down regulation of the apelin pathway might conceivably contribute to the pathophysiology of heart failure and suggests therapeutic potential for apelin receptor agonism. To date there have been no reports of the effects of apelin in patients with heart failure. Encouragingly however, the cardiovascular effects of exogenous apelin are maintained or even increased in rodents with heart failure suggesting, scope to augment endogenous apelin signalling.

5.5.1 STUDY LIMITATIONS

We acknowledge that this study has only assessed the acute effects of apelin administration. Our data on the direct cardiac effects of apelin are not definitive and we believe that further work is needed to assess the load-independent effects of apelin on myocardial contractility. To determine whether apelin causes load-independent increases in myocardial contractility is challenging in a clinical study and would require the use of a conductance catheter with manipulation of cardiac filling pressures.

5.5.2 CONCLUSIONS

Consistent with its effects in preclinical models, we have shown that acute apelin administration causes peripheral vasodilatation, augments cardiac contractility and output and reduces left ventricular preload and afterload. If replicated in suitable patient populations, this profile of cardiovascular actions would suggest a potential therapeutic role for apelin receptor agonism in heart failure.

CHAPTER 6

CARDIOVASCULAR RESPONSES TO APELIN IN PATIENTS WITH CHRONIC HEART FAILURE

Japp AG, Cruden NL, Barnes G *et al.*
Acute cardiovascular effects of apelin in humans:
potential role in patients with chronic heart failure.
Circulation 2010;**121**:1818-1827.

6.1 SUMMARY

The apelin system mediates important cardiovascular effects in preclinical and clinical models. The study objectives were to establish the direct vascular and systemic haemodynamic effects of acute apelin agonism in patients with stable chronic heart failure and matched healthy control subjects. Forearm blood flow was measured by venous occlusion plethysmography during intrabrachial infusions of (Pyr^{1})apelin-13 (0.3, 1, 3 nmol/min), acetylcholine (5, 10, 20 μg/min) and SNP (1, 2, 4 μg/min) in 12 patients and 12 controls. Cardiac output, peripheral vascular resistance, blood pressure and heart rate were assessed by thoracic bioimpedance and sphygmomanometry during intravenous infusions of (Pyr^{1})apelin-13 (30, 100, 300 nmol/min) and matched saline placebo in 8 patients and 8 controls. (Pyr^{1})apelin-13, acetylcholine and SNP caused forearm vasodilatation in patients and controls (all $P<0.0001$). Vasodilatation to acetylcholine ($P=0.01$) but not apelin ($P=0.3$) or SNP ($P=0.9$) was attenuated in patients with heart failure. Systemic infusions of (Pyr^{1})apelin-13 (30-300 nmol/min) increased cardiac index and lowered mean arterial pressure and peripheral vascular resistance in patients and controls (all $P<0.01$) but increased heart rate only in controls ($P<0.01$). The direct vascular and systemic haemodynamic effects of apelin are preserved in patients with stable chronic heart failure. Apelin receptor agonism represents a novel potential therapeutic target for patients with heart failure.

6.2 INTRODUCTION

Apelin and the apelin receptor (previously known as 'APJ') constitute a recently discovered peptidic system with an emerging role in cardiovascular regulation [O'Dowd *et al* 1993; Tatemoto *et al* 1998; Pitkin *et al* 2010a] and potential therapeutic application in heart failure.

Mice with deletion of the apelin gene develop premature heart failure unless plasma apelin concentrations are restored [Kuba *et al* 2007]. In healthy rodents, exogenous apelin potently enhances myocardial contractility without inducing left ventricular hypertrophy [Ashley *et al* 2005] and achieves this whilst simultaneously reducing ventricular preload and afterload. These effects are maintained in preclinical models of heart failure: *in vitro*, apelin increases contractility in the failing myocardium to the same [Farkasfalvi *et al* 2007] or greater [Dai *et al* 2006] extent as in the normal myocardium and, *in vivo*, acute apelin infusion restores ejection fraction, increases cardiac output and reduces LVEDP in rats with chronic heart failure [Berry *et al* 2004; Jai *et al* 2006].

We recently provided the first evidence that apelin has cardiovascular actions *in vivo* in man [Japp *et al* 2008]. In healthy volunteers, apelin causes nitric oxide-mediated peripheral vasodilatation and increases cardiac output whilst, in patients undergoing elective coronary angiography, it reduces left ventricular preload and increases cardiac contractility.

Paralleling findings in rodent models, failing human hearts exhibit altered expression patterns of apelin and its receptor [Foldes *et al* 2003] whilst patients with severe chronic heart failure have reduced plasma apelin levels [Chong *et al* 2006]. The impact of these changes on apelin-mediated biological effects has not previously been investigated. Therefore, as a first step to exploring the therapeutic potential of apelin receptor agonism in heart failure, we sought to determine the local vascular, systemic haemodynamic and neurohormonal effects of acute apelin administration in patients with stable chronic heart failure.

6.3 METHODS

All studies were performed with the written informed consent of volunteers, the approval of the local Research Ethics Committee and in accordance with the Declaration of Helsinki.

6.3.1 SUBJECTS

Patients with heart failure were eligible for inclusion if they had stable NYHA class II-IV symptoms, were on maximally tolerated doses of heart failure medication for at least 3 months, and had objective evidence of left ventricular impairment (left ventricular end-diastolic diameter >5.5 cm and left ventricular ejection fraction <40% or shortening fraction <20%) [Cruden *et al* 2004]. Patients were excluded if they had haemodynamically significant valvular heart disease, renal or hepatic failure, or had previous malignant ventricular arrhythmias. Control volunteers had no history of any clinically significant medical condition or symptoms of recent illness

and avoided vasoactive and non-steroidal anti-inflammatory drugs for 7 days prior to studies. Patients withheld their usual medications on the day of the study until completion of the study protocol and all participants abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least 4 hours before each study.

6.3.2 DRUGS

The effects of apelin receptor agonism were assessed using synthetic pharmaceutical grade (Pyr^{1})apelin-13. Acetylcholine and SNP were administered as endothelium-dependent and independent control vasodilators respectively.

6.3.3 MEASUREMENTS

All studies were carried out in a quiet, temperature-controlled room (23-25°C) with subjects in the supine position.

Peripheral Arterial Studies

Subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions and the rate of infusion was kept constant at 1 mL/min. Blood flow was measured in the infused and non-infused forearms by bilateral forearm venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [Newby *et al* 1997a; Newby *et al* 1999].

Systemic Haemodynamic Studies

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms to allow drug infusion and sampling of venous blood. Blood pressure and heart rate were recorded with a semi-automated non-invasive oscillometric sphygmomanometer; mean arterial pressure was defined as the sum of the diastolic blood pressure and a third of the pulse pressure. Cardiac output was measured non-invasively using a TEB monitor [Northridge *et al* 1990] following manufacturer's guidelines and corrected for body surface area to give cardiac index. At each time point, cardiac output was taken as the mean of three recordings, each recording representing the average of 16 consecutive heartbeats. Peripheral vascular resistance index was calculated as mean arterial pressure divided by cardiac index and expressed in dyne.s/cm^5/m^2. Continuous electrocardiographic monitoring was performed during all studies.

Assays

Blood samples (10 mL) were drawn into tubes containing either EDTA or lithium heparin, and kept on ice before centrifugation at 2000 *g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma renin activity was measured under standard conditions through the generation of angiotensin I as determined by radioimmunoassay [Haber *et al* 1969]. Plasma BNP and AVP concentrations were measured by immunoradiometric assay and direct radioimmunoassay respectively.

6.3.4 PROTOCOL DESIGN (Figure 6.1)

Protocol 1: Peripheral arterial studies

Twelve patients with heart failure and 12 age- and sex-matched healthy controls attended on a single occasion and had blood drawn for baseline measurement of BNP and plasma renin activity. In a double-blinded randomised manner, all subjects then received intrabrachial infusions of (Pyr^{1})apelin-13 (0.3, 1, 3 nmol/min) [Japp *et al* 2008], acetylcholine (5, 10, 20 μg/min) [Japp *et al* 2008] and SNP (1, 2, 4 μg/min) [Taddei *et al* 1998] for 6 minutes at each dose with drugs separated by a 20-minute saline infusion. Blood flow was measured at 6-minute intervals throughout the study in the infused and non-infused forearms by venous occlusion plethysmography.

Protocol 2: Systemic haemodynamic studies

Eight patients with heart failure and eight healthy matched controls attended on a single occasion. In a double-blind randomised manner, intravenous (Pyr^{1})apelin-13 (30, 100, 300 nmol/min) and matched saline placebo were infused for 5 minutes at each dose. Apelin and saline infusions were separated by a 30-minute saline infusion. Haemodynamic recordings were made at 10-minute intervals prior to commencement of drug infusion and at 5-minute intervals thereafter. Venous blood samples were obtained at baseline and at 5, 15 and 30 minutes post drug infusion for measurement of BNP, AVP and plasma renin activity.

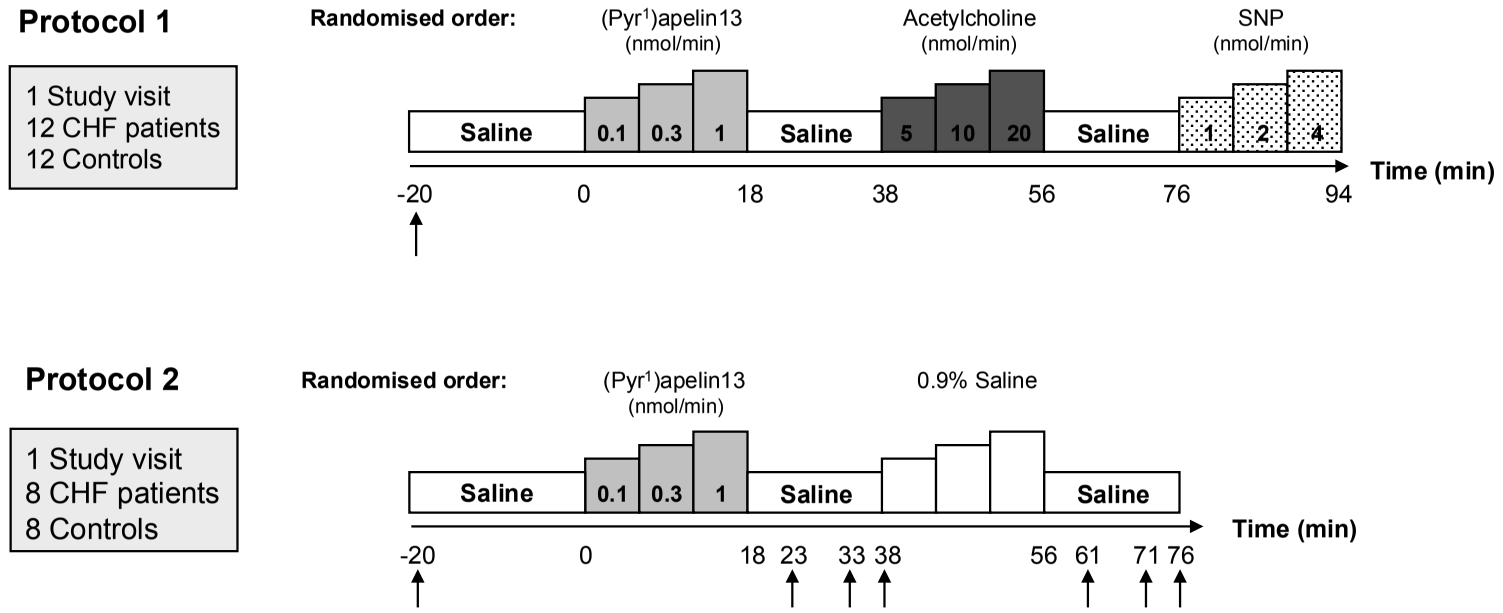

Figure 6.1 Study design. Protocol 1: Intrabrachial infusions; Protocol 2: Intravenous infusions; with venous blood sampling (↑).
SNP - sodium nitroprusside; CHF - chronic heart failure.

6.3.5 DATA AND STATISTICAL ANALYSES

Forearm and systemic haemodynamic variable were analysed as described previously [Newby *et al* 1997a, Leslie *et al* 2005]. Plasma renin activity and BNP concentrations were not normally distributed and were therefore log transformed prior to statistical analysis. In order to minimise the effects of intergroup variability in baseline haemodynamic and blood flow variables, responses to drug infusion are expressed as percentage change from baseline. Variables are reported as mean ± SEM and, where appropriate analysed using repeated measures ANOVA with post-hoc Bonferroni corrections and two-tailed Student's *t*-test as appropriate. Statistical significance was taken at the 5% level.

6.4 RESULTS

All studies were well tolerated with no serious adverse events or ECG changes during apelin administration. Patients with heart failure were predominantly male and middle-aged with the aetiology attributable to ischaemic heart disease or dilated cardiomyopathy (Table 6.1). They were well treated with optimal or maximally tolerated heart failure therapy and had mild to moderate symptoms with a low ejection fraction and elevated BNP concentrations and plasma renin activity (Table 6.1).

Peripheral Blood Flow

There was an apparent trend towards lower basal FBF in patients with heart failure, compared with controls but this did not reach statistical significance (Table 6.1).

TABLE 6.1 Baseline characteristics of patients with heart failure and healthy controls

	Peripheral Arterial Studies		Systemic Haemodynamic Studies	
	Controls (n=12)	Patients (n=12)	Controls (n=8)	Patients (n=8)
Age (years)	60 ± 2	61 ± 3	57 ± 3	55 ± 4
Sex (M/F)	3/9	2/10	8/8	8/8
BMI (kg/^{2})	27 ± 1	27 ± 1	25 ± 1	29 ± 2*
LVEDD (cm)	-	7.5 ± 0.6	-	6.5 ± 0.5
EF (%)	-	27 ± 3	-	28 ± 4
NYHA (II / III)	-	10/2	-	4/4
Aetiology (IHD / DCM)	-	6/6	-	5/3
Medication:				
ACEi / ARB	0/12	12/12	0/8	7/8
Beta-blocker	0/12	8/12	0/8	8/8
Diuretic	0/12	1s2/12	0/8	7/8
Aldosterone antagonist	0/12	6/12	0/8	4/8
Baseline:				
Heart rate (bpm)	66 ± 3	68 ± 2	58 ± 3	71 ± 4*
MAP (mmHg)	98 ± 3	96 ± 2	92 ± 3	90 ± 4
Infused FBF	3.2 ± 0.4	2.6 ± 0.3	-	-
Non-infused FBF	3.0 ± 0.4	2.4 ± 0.3	-	-
CI (L/min/m^2)	-	-	3.3 ± 0.5	2.7 ± 0.6
PVRI (dyne.s/cm^5/m^2)	-	-	2423 ± 290	2993 ± 549
BNP (pg/mL)	10 ± 4	237 ± 59†	6 ± 2	215 ± 161*
PRA (ng/mL)	1.5 ± 0.2	30.1 ±10.6†	1.5 ± 0.4	30.4 ± 10.5†
AVP (pg/mL)	-	-	3.0 ± 1.9	1.9 ± 0.5

Data expressed as number of patients or mean±SEM; *P<0.05, †P<0.01 *versus* controls.

BMI - body mass index; LVEDD - left ventricular end-diastolic diameter; EF - ejection fraction; NYHA - New York Heart Association; IHD - ischaemic heart disease; DCM - dilated cardiomyopathy; ACEi - angiotensin-converting enzyme inhibitor; ARB - angiotensin receptor blocker; MAP - mean arterial pressure; FBF - forearm blood flow; CI - cardiac index; PVRI - peripheral vascular resistance index; BNP - brain natriuretic peptide; PRA - plasma renin activiy; AVP - arginine vasopressin; SEM - standard error of the mean.

There was no significant change in heart rate, blood pressure or non-infused FBF in either patients or controls. In the infused arm, (Pyr^{1})apelin-13, acetylcholine and SNP caused vasodilatation (P<0.001) in both healthy volunteers and patients with heart failure (Figure 6.2). Whilst vasodilatation to (Pyr^{1})apelin-13 and SNP was similar between the groups, vasodilatation to acetylcholine was reduced in patients with heart failure.

Systemic Haemodynamic Studies

Compared with saline placebo, intravenous administration of (Pyr^{1})apelin-13 caused an increase in cardiac output, and reductions in blood pressure and peripheral vascular resistance in both patients and controls as well as an increase in heart rate in controls (P<0.01 for all; Figure 6.3 and Table 6.2). There were no differences in the responses to apelin between patients with heart failure and controls, with the exception of heart rate, in which a greater response to apelin was observed in controls (P<0.05; Figure 6.4).

At baseline, plasma BNP concentration and plasma renin activity were both higher in patients compared with controls (both P<0.01) whilst plasma AVP concentration did not differ significantly between the two groups (P=0.24; Table 6.1). There was no change in any of these variables in either group at 5, 15 and 30 minutes after apelin infusion (Figure 6.5).

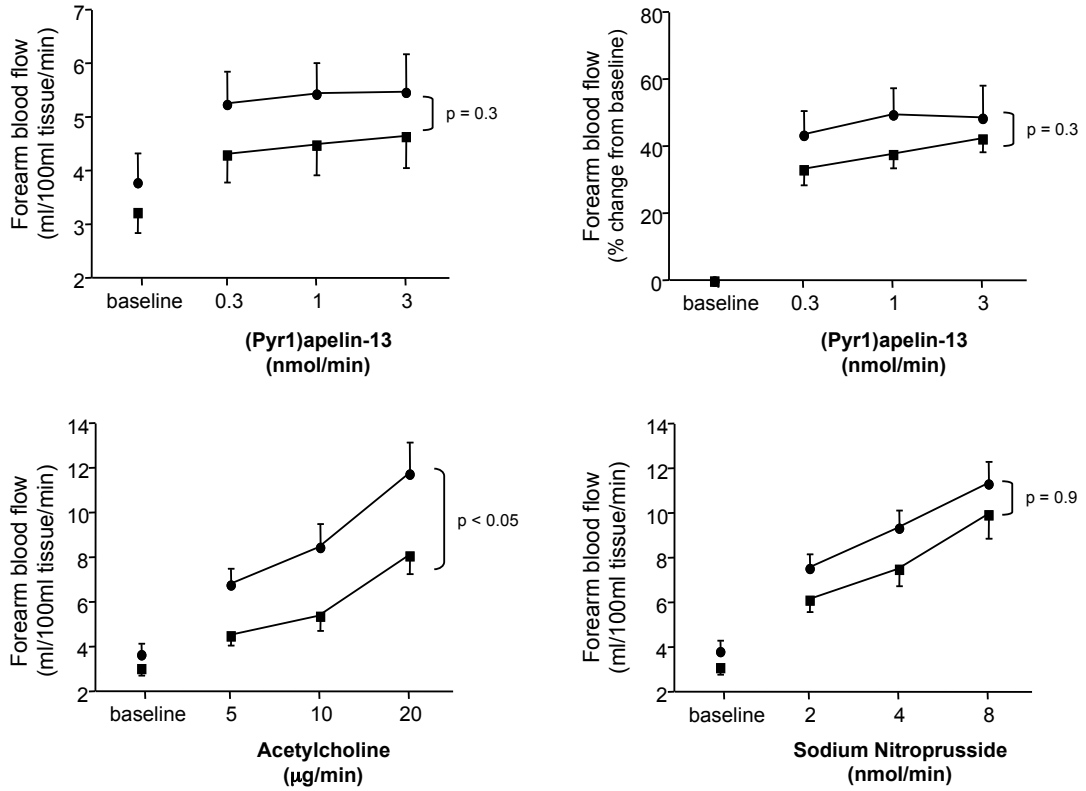

Figure 6.2 Infused forearm blood flow in patients with chronic heart failure (■) and matched controls (●) during intrabrachial infusion of (Pyr^1)apelin-13, acetylcholine and SNP: for all dose-responses P<0.001 (ANOVA); for patients *versus* controls (Pyr^1)apelin-13 (P=0.3), acetylcholine (P<0.05) and SNP (P=0.9).
SNP - sodium nitroprusside; ANOVA - analysis of variance.

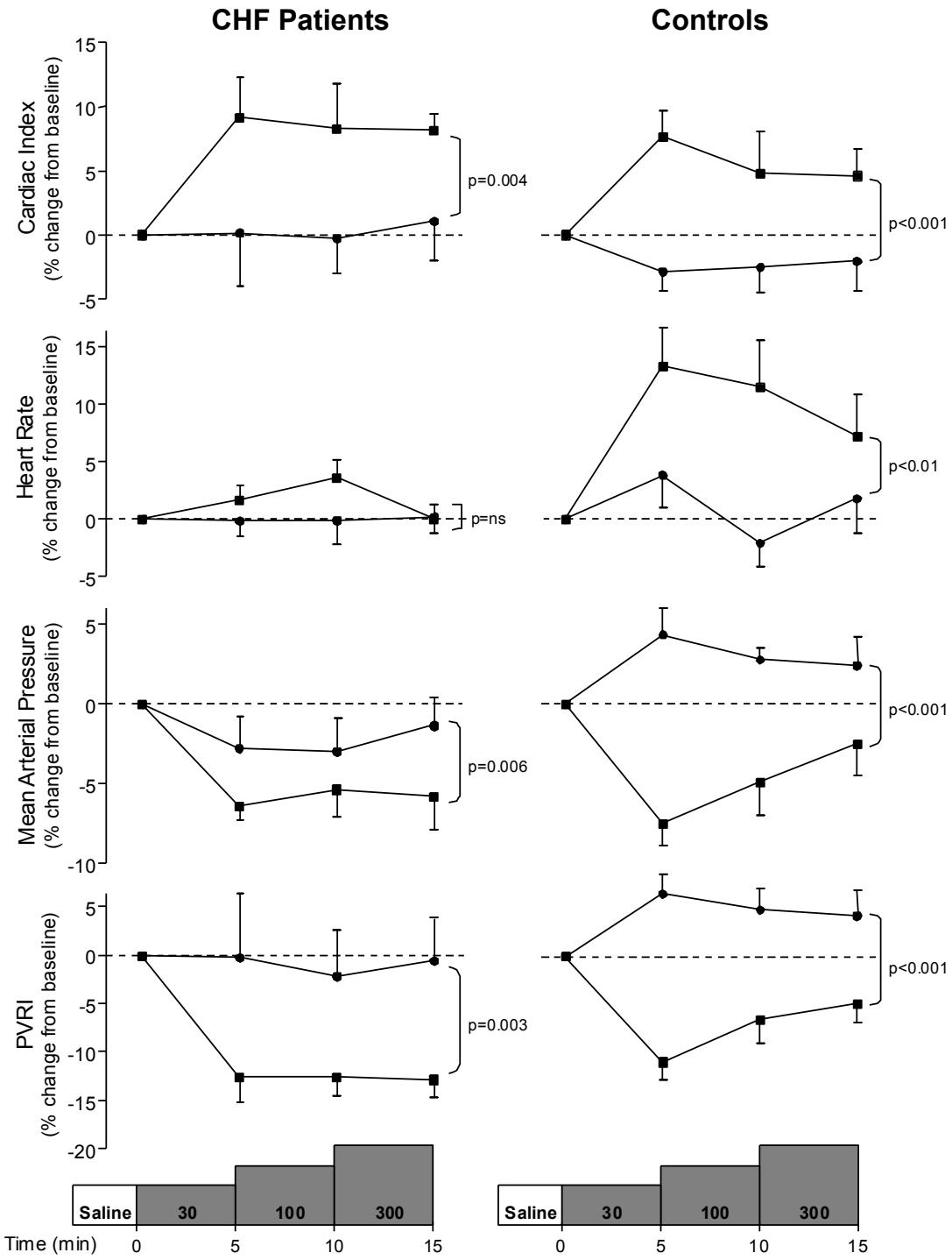

Figure 6.3 Percentage change from baseline in mean arterial pressure, heart rate, cardiac index and peripheral vascular resistance index during infusion of (Pyr[1])apelin-13 (■) and saline placebo (●) in patients with chronic heart failure (left hand panel) and matched controls (right hand panel): for all dose-responses, P<0.01 compared with saline placebo (two-way ANOVA), except change in heart rate in patients (P=ns)

ANOVA - analysis of variance; ns - not significant; CHF - chronic heart failure; PVRI - peripheral vascular resistance index.

TABLE 6.2 Systemic haemodynamic variables during intravenous apelin infusion

	Baseline	Apelin (nmol/min)				Washout	
		30	100	300	5 min	10 min	15 min
CHF Patients							
Mean arterial pressure (mmHg)	93±5	87±4*	88±3	87±3*	92±4	92±4	90±3
Heart rate (bpm)	72±4¥	73±3	74±4	71±3	70±4	70±4	70±3
Cardiac Index ($L/min/m^2$)[§]	2.6±0.4	2.8±0.4	2.9±0.5*	2.9±0.4	2.7±0.4	2.7±0.5	2.7±0.5
PVRI ($dyne.s/cm^5/m^2$)[§]	2993±549	2579±436†	2620±498†	2583±449†	2887±547	2862±552	2884±660
Controls							
Mean arterial pressure (mmHg)	95±3	89±3†	91±3	93±2	98±2	93±3	97±2
Heart rate (bpm)	56±2	63±3†	62±2†	60±2	57±3	59±2	61±1
Cardiac Index ($L/min/m^2$)	3.2±0.4	3.4±0.4*	3.4±0.4	3.3±0.4	3.2±0.4	3.2±0.5	3.1±0.4
PVRI ($dyne.s/cm^5/m^2$)	2495±271	2162±223†	2288±230*	2328±213	2557±221	2481±274	2668±293

Data expressed as mean±SEM. ANOVA (*versus* baseline), †P<0.01, *P<0.05; Student's *t*-test (patient *versus* control), ¥P<0.001. [§]n=5 due to technical failure of bioimpedance. PVRI indicates peripheral vascular resistance index.
CHF - chronic heart failure; PVRI - peripheral vascular resistance index; SEM - standard error of the mean; ANOVA - analysis of variance.

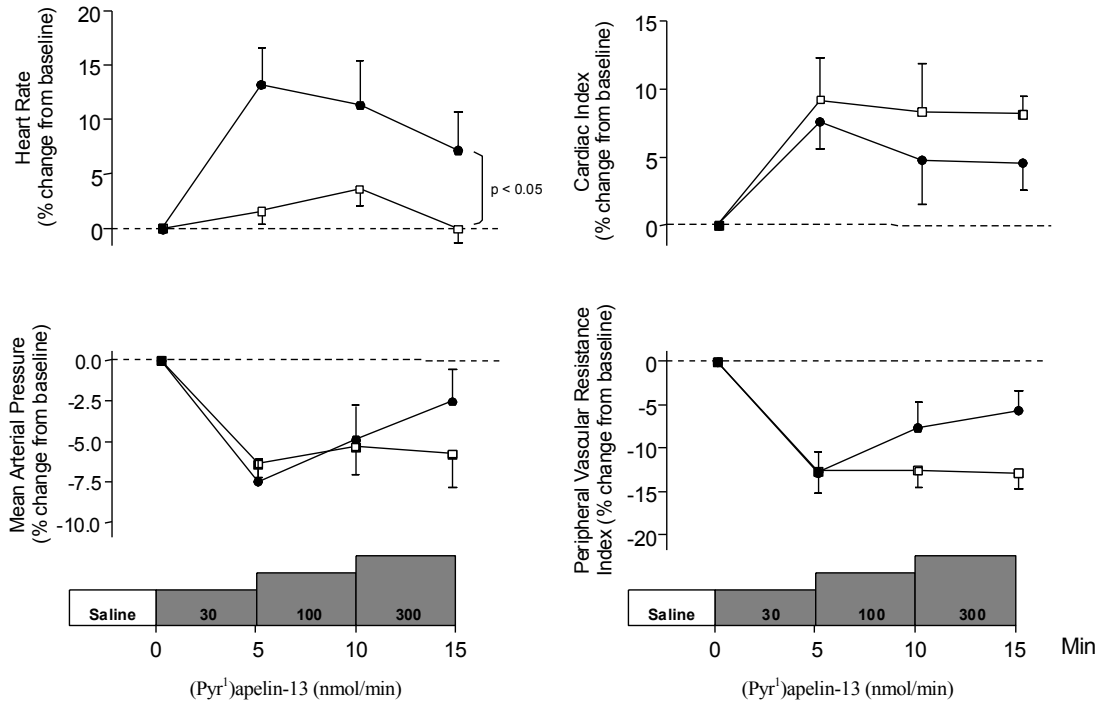

Figure 6.4 Percentage change from baseline in mean arterial pressure, heart rate, cardiac index and peripheral vascular resistance index during infusion of (Pyr[1])apelin-13 in patients with chronic heart failure (□) and matched controls (●). Two-way ANOVA (patients *versus* controls) heart rate, P<0.05; mean arterial pressure, cardiac index and peripheral vascular resistance index, P=ns.
ANOVA - analysis of variance; ns - not significant.

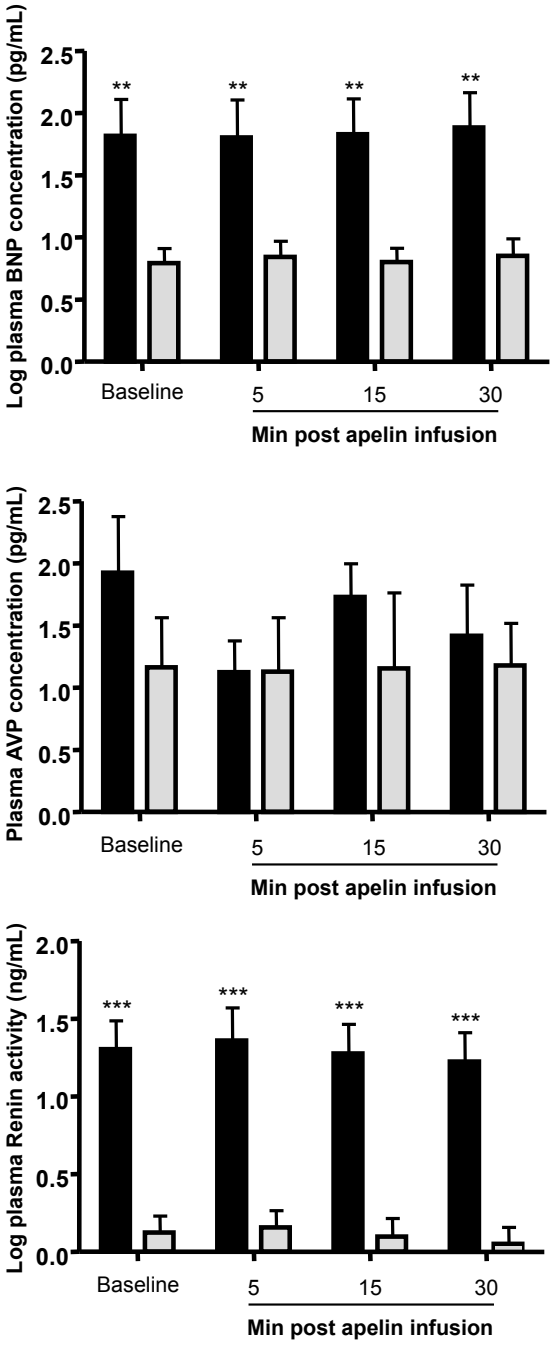

Figure 6.5 Plasma BNP and AVP concentrations and plasma renin activity in venous blood samples from heart failure patients (■) and matched controls (■) before and after infusion of (Pyr^1)apelin-13. **P<0.01, ***P<0.001, two-way ANOVA (patients *versus* controls) with post-hoc Bonferroni tests. BNP - brain natriuretic peptide; AVP - arginine vasopressin; ANOVA - analysis of variance.

6.5 DISCUSSION

In both preclinical and clinical models, the apelin system mediates important cardiovascular effects, reducing peripheral vascular resistance through nitric oxide-mediated arterial vasodilatation whilst increasing cardiac contractility and output. We here report that the direct vascular and systemic haemodynamic effects of apelin are preserved in patients with stable chronic heart failure. We conclude that apelin receptor agonism has potential therapeutic benefits in patients with heart failure maintained on optimal contemporary medical therapy.

We have previously shown, in keeping with data from animal and *ex vivo* models, that apelin causes vasodilatation *in vivo* in the human forearm circulation through predominantly nitric oxide-dependent mechanisms [Salcedo *et al* 2007]. In these studies we extend this work to demonstrate that apelin-induced vasodilatation is preserved in patients with heart failure. Whilst the study may have been underpowered to detect small intergroup differences, we saw no evidence of a major attenuation of vasodilatation to apelin in patients with heart failure. This is perhaps surprising as the same patients demonstrated reduced vasodilatation to acetylcholine that, like apelin, relies predominantly on endothelium-dependent pathways. One possible explanation is that alterations in endogenous plasma apelin concentrations [Chong *et al* 2006] or apelin receptor expression [Foldes *et al* 2003] in the setting of heart failure may augment responses to exogenous apelin and thereby offset any diminution of endothelial function. On the other hand, it is controversial whether patients with stable treated heart failure have evidence of major endothelial

dysfunction [Negrao *et al* 2000] especially when maintained on optimal medical therapy. Given the rapidity of its metabolism, the reduced vasodilatation to acetylcholine in patients with heart failure may be due to lower basal blood flow rather than endothelial dysfunction *per se* [Chowienczyk *et al* 1994].

In both patients with chronic heart failure and healthy adults, systemic apelin administration causes a reduction in peripheral vascular resistance accompanied by an increase in cardiac output. In a previous study in young healthy subjects (aged 21-27 years), heart rate increased slightly while blood pressure was maintained [Japp *et al* 2008]. In an older cohort of healthy subjects in this study we observed a similar increase in heart rate with a transient drop in blood pressure. Finally, in heart failure patients, apelin elicited no change in heart rate but a sustained reduction in blood pressure. We believe the most likely explanation for these differing patterns of heart rate and blood pressure response to apelin relates to blunting of reflex sympathetic activation and other neurohormonal responses to peripheral vasodilatation, caused by ageing, drug therapy (particularly beta-blockade) and, possibly, heart failure *per se*.

Consistent with our previous study in healthy volunteers, the changes in haemodynamic variables in both patients and controls, failed to exhibit a clear dose-response relationship. This was also seen in our previous studies in the forearm circulation where vasodilator responses to apelin plateaued early despite repeated dose escalation [Japp *et al* 2008]. In the same model, continuous infusion of (Pyr^{1})apelin-13 for 42 minutes elicited sustained, near-maximal vasodilatation

suggesting that this plateauing of effect was due to a ceiling of apelin-mediated vasodilatation rather than rapid apelin receptor desensitisation. Accordingly, the flat dose-response to apelin in these studies is unlikely to represent tachyphylaxis but may reflect our selection of doses or an 'all-or-nothing' response.

The mechanisms by which apelin increases cardiac output in man have yet to be fully established. These and our previous studies strongly suggest a contribution from peripheral arterial vasodilatation. However, the possibility of an additional direct effect on myocardial contractility is suggested by the potent inotropic effects of apelin *in vitro* [Szokodi *et al* 2002] and the load-independent increases in cardiac contractility observed *in vivo* in rodent models [Ashely *et al* 2005]. Whilst we have previously demonstrated an increase in cardiac contractility following intracoronary apelin administration in man, we were unable to infer a direct inotropic effect due to concomitant changes in loading conditions suggesting overspill of intracoronary apelin in to the peripheral circulation and consequent systemic vascular effects. In support of an additional direct cardiac action, recent *in vitro* studies have demonstrated, for the first time, that apelin has inotropic effects in human cardiac tissue. Nonetheless, a direct effect of apelin on myocardial contractility *in vivo* in man has yet to be demonstrated conclusively.

Although apelin exerts similar effects to BNP on coronary blood flow, systemic haemodynamics and fluid balance, the lack of rise in plasma BNP following apelin administration confirms that its acute cardiovascular effects are not mediated via BNP release. Whether chronic apelin administration alters plasma BNP levels in

patients with chronic heart failure will be an important research question for future studies exploring the potential therapeutic effects of apelin receptor agonism.

In preclinical models, apelin opposes the actions of AVP on fluid homeostasis and, when administered centrally, inhibits AVP release [De Mota *et al* 2004]. Mounting evidence also suggests a direct counter-regulatory role for apelin in relation to the renin-angiotensin system [Ashley *et al* 2006; Chun *et al* 2008]. In these studies we observed no changes in plasma renin activity or AVP concentration for up to 30 minutes following peak apelin infusion suggesting that apelin does not inhibit acute secretion of renin or AVP under normal physiological conditions or in compensated heart failure in man. However further dedicated studies are required to explore potential functional interactions between these systems *in vivo* over a broader time frame and under varying physiological conditions.

In considering apelin as a potential therapeutic target for patients with heart failure, the haemodynamic profile of action described above is broadly favourable. Some established inotropic therapies have been associated with adverse outcomes in this patient group and, in particular, agents that act by increasing intracellular calcium concentrations tend to increase myocardial oxygen consumption, exert pro-arrhythmic effects and promote left ventricular hypertrophy. However, in preclinical models, apelin appears to exert its inotropic effects at least in part through increasing myofilament sensitivity to calcium and does not induce left ventricular hypertrophy, even during chronic administration. In addition, its ability to reduce cardiac loading conditions may serve to limit cardiac work and myocardial oxygen consumption.

There are now several reports of reduced plasma apelin concentrations in patients with heart failure [Japp and Newby 2008] suggesting scope to augment apelin activity. By demonstrating preserved local and systemic cardiovascular responses to exogenous apelin in patients with heart failure, we here confirm that the signalling capacity of apelin receptors is not exhausted by endogenous apelin in this patient cohort - an essential prerequisite for therapeutic strategies employing apelin receptor agonism.

The clinical relevance of our findings is enhanced by the inclusion of patients maintained on existing evidence-based pharmacological therapies. This is particularly important given the recent emergence of direct interactions between the renin-angiotensin and apelin systems including direct antagonism of angiotensin II by apelin [Iwanaga *et al* 2006; Chun *et al* 2008]. As all but one of our patients with heart failure were receiving treatment with ACE inhibitors or angiotensin receptor blockers, our findings imply a role for apelin in cardiovascular regulation independent of angiotensin II signalling pathways and suggest potential for pharmacological synergism through combined apelin receptor agonism and renin-angiotensin system inhibition.

We acknowledge that this study has only assessed the acute effects of apelin administration. We cannot reliably infer the consequences of chronic apelin administration in patients with chronic heart failure. However, our study would suggest that apelin infusion might be of benefit in patients with acute decompensated heart failure where these haemodynamic effects of vasodilatation, increased cardiac

output, reduced preload, and potential inotropy would be beneficial.

6.5.1 CONCLUSION

To date, both preclinical and limited clinical studies suggest that the apelin system may have an important role in the pathogenesis of heart failure. Although the apelin pathway appears to be down regulated in cardiac failure, we have shown that the favourable haemodynamic actions of apelin persist and are additive to the effects of renin-angiotensin system inhibition. Apelin receptor agonism may therefore provide a new therapeutic target in the treatment of acute and chronic heart failure.

CHAPTER 7

CONCLUSIONS

7.1 INTRODUCTION

Heart failure constitutes a major and growing health burden in developed nations. Despite considerable treatment advances over the past two decades, it has a prognosis worse than that of many cancers and results in severe morbidity with impaired quality of life and recurrent hospitalisation [Young *et al* 2004; Cleland *et al* 2005; Mosterd and Hoes 2007]. The development of novel treatments for patients with heart failure therefore remains a major priority. G protein-coupled receptors play an essential role in the physiological control of the cardiovascular system and represent the greatest target for existing pharmacological treatments [Foord *et al* 2005; Maguire and Davenport 2005]. Many of the recent pharmacological advances in the treatment of heart failure, including AT_1 and beta-adrenergic receptor blockers, have arisen through the specific targeting of G protein-coupled receptor systems, and have provided additive incremental morbidity and mortality benefit [CIBIS-II Investigators 1999; MERIT-HF Study Group 1999; Packer *et al* 2001; McMurray *et al* 2003].

In 1993 a novel G protein-coupled receptor, originally called 'APJ' and since renamed the 'apelin receptor' was identified through reverse homology cloning [O'Dowd *et al* 1993] and subsequently paired with its endogenous ligand, apelin [Tatemoto *et al* 1998]. In preclinical models, exogenous apelin mediates nitric oxide-dependent vasodilatation, reduces ventricular preload and afterload, and potently increases myocardial contractility without inducing left ventricular hypertrophy [Tatemoto *et al* 2001, Ashley *et al* 2005]. In rodents the endogenous apelin system is

up regulated by hypoxia [Ronkainen *et al* 2007; Sheikh *et al* 2008] and pressure overload [Iwanaga *et al* 2006] and apelin signalling appears to be critical in maintaining cardiac function during cardiovascular stress [Kuba *et al* 2007; Charo *et al* 2009]. However expression of the apelin system is reduced in both animal models of heart failure [Iwanaga *et al* 2006; Jia *et al* 2006] and patients with chronic heart failure [Földes *et al* 2003; Chong *et al* 2006], and this down regulation appears to parallel the deterioration in cardiac performance. The down regulation of the apelin system in heart failure may therefore exacerbate myocardial dysfunction and represent a further maladaptive neurohormonal response in addition to over-stimulation of the renin-angiotensin-aldosterone and adrenergic systems. Importantly, the beneficial effects of exogenous apelin on cardiac contractility and loading conditions are maintained in preclinical models of heart failure [Berry *et al* 2004; Atluri *et al* 2007; Farkasfalvi *et al* 2007]. This confirms that, irrespective of changes in apelin system expression, the signalling capacity of the apelin receptor is not exhausted by endogenous apelin and provides a powerful rationale for exploring the therapeutic potential of apelin receptor agonism in patients with heart failure.

To date, the cardiovascular effects of apelin receptor agonism *in vivo* in man have not been established and the therapeutic potential of this strategy cannot be realised without detailed clinical investigation. Through a series of clinical studies combing local and regional infusions of two apelin peptides with robust and well-validated techniques, I have addressed this important research priority. Specifically, I have established the *in vivo* effects of apelin receptor agonism in man on venous and peripheral resistance vessel tone, coronary blood flow and cardiac and systemic

haemodynamics. Additionally I have compared the direct vascular and systemic haemodynamic effects of the two principal biologically active apelin receptor ligands in man and examined the endothelial mechanisms by which apelin evokes its direct vascular actions. Having demonstrated these effects in healthy volunteers I have then examined cardiovascular responses to apelin receptor agonism in patients with chronic heart failure and compared these to age- and sex-matched healthy controls.

7.2 THE DIRECT VASCULAR ACTIONS OF APELIN *IN VIVO* IN MAN

Ex vivo, apelin causes endothelium-dependent vasorelaxation in human mesenteric artery [Salcedo *et al* 2007] and direct vasconstriction in endothelium-denuded human saphenous vein and mammary artery [Katugampola *et al* 2001; Maguire *et al* 2009]. In rodent models, *in vivo*, the predominant vascular effect is vasodilatation in the arterial and venous circulation [Tatemoto *et al* 2001; Cheng *et al* 2003]. In this thesis I have for the first time explored the *in vivo* effects of apelin in the human vasculature. Specifically, I sought to determine its direct vasomotor effects in the peripheral venous and peripheral and coronary arterial circulation.

Local infusion of apelin-36 or (Pyr^{1})apelin-13 had no effect on either basal or preconstricted dorsal hand vein diameter suggesting that apelin does not modulate peripheral venous tone *in vivo* in man. However superficial skin veins are not representative of other venous beds and are of less haemodynamic importance than deep capacitance veins [Schmitt *et al* 2002]. During subsequent invasive studies, I observed a reduction in LVEDP following apelin administration. This finding is

consistent with preclinical data in rodents and provides indirect evidence that apelin acts as a venodilator, effecting a net reduction in systemic venous tone.

In contrast to the lack of effect in hand veins, I have demonstrated that apelin causes reproducible and sustained vasodilation in the human arterial forearm circulation. The vasodilator response to apelin exhibited a rapid onset and a slow offset with effects persisting for up to 42 minutes after cessation of infusion. By measuring local plasma apelin concentrations, I was able to confirm that this effect was not due to persistence of circulating apelin. Whilst some *in vitro* responses to apelin exhibit desensitisation, forearm vasodilatation was sustained with no evidence of tachyphylaxis during a continuous 42-minute infusion.

Having established the vasoactive effects of apelin in the human forearm circulation, I next sought to determine the contribution of the endothelium-derived vasodilators nitric oxide and prostacyclin to apelin-mediated vasodilatation. Utilising the nitric oxide clamp, I have demonstrated that apelin-mediated vasodilatation *in vivo* is mediated predominantly through nitric oxide. In contrast there was no discernible contribution from prostacyclin generation. This is in keeping with myography studies in human mesenteric artery where apelin-mediated vasorelaxation was attenuated by nitric oxide synthase but not cyclooxygenase inhibition [Salcedo *et al* 2007]. Recently apelin has been reported to cause prostacyclin-dependent vasorelaxation *ex vivo* in human mammary artery from patients undergoing coronary artery bypass surgery [Maguire *et al* 2009]. Further work is therefore required to determine whether prostacyclin contributes to apelin-mediated vasodilatation *in vivo* in subjects

with established endothelial dysfunction. Although apelin is capable of inducing vasoconstriction in endothelium-denuded vessels *in vitro* through a direct effect on vascular smooth muscle, there are no reports of vasoconstriction *in vivo*. Correspondingly, I have demonstrated that in the healthy human vasculature, the predominant vascular effect of apelin *in vivo* is vasodilatation even in the presence of combined prostacyclin and nitric oxide inhibition.

Establishing the *in vivo* effects of apelin on coronary blood flow is critical to determining the potential therapeutic application of apelin receptor agonism. Consistent with its effects in the forearm arterial circulation, I have demonstrated that apelin is a direct coronary vasodilator in man. Apelin has recently been reported to cause vasoconstriction *in vitro* in human atherosclerotic coronary artery [Pitkin *et al* 2010b]. The patients in my studies were free of significant coronary artery disease and hence the *in vivo* vasomotor effects of apelin on atherosclerotic coronary arteries have yet to be established.

7.3 THE CARDIAC AND SYSTEMIC HAEMODYNAMIC EFFECTS OF APELIN IN MAN

In keeping with findings in preclinical models, I have shown that apelin administration in man increases cardiac contractility and output. The concomitant reduction in peripheral vascular resistance and transient rise in heart rate during intravenous apelin infusion suggest that this effect is mediated, at least in part, by reduced afterload and reflex sympathetic activation secondary to arterial

vasodilatation. However an additional direct myocardial action is suggested by the potent inotropic effects of apelin in human cardiac tissue *ex vivo*. Consistent with this, I have demonstrated an increase in left ventricular dP/dtmax following intracoronary apelin injection. However, dP/dtmax is sensitive to ventricular loading conditions which, in these studies, were also altered indicating likely spillover of apelin from the coronary circulation into the peripheral circulation and consequent systemic vascular effects. Therefore this thesis does not provide definitive evidence of a direct effect of apelin on myocardial contractility *in vivo* in man.

In accordance with preclinical data and my findings in the forearm arterial circulation, systemic apelin administration reduces peripheral vascular resistance and, in older adults, causes a transient reduction in mean arterial pressure. Moreover, intracoronary administration reduces left ventricular end-diastolic and peak pressure. These results demonstrate, for the first time, that the direct vascular actions of apelin in man translate into alterations in systemic haemodynamics and left ventricular loading conditions and provide the first evidence of apelin-mediated vasodilatation in the human venous circulation.

7.4 CARDIOVASCULAR RESPONSES TO APELIN IN PATIENTS WITH CHRONIC HEART FAILURE

The demonstration in Chapters 3-5 of this thesis that exogenous apelin administration in man causes peripheral and coronary arterial vasodilatation, augments cardiac contractility and output and reduces left ventricular preload and

afterload suggested potential therapeutic application in heart failure. Accordingly, I sought to determine cardiovascular responses to apelin receptor agonism in patients with stable chronic heart failure and to compare these with age- and sex-matched controls. Consistent with preclinical findings *in vitro* and *in vivo*, I have shown that the direct vascular and systemic haemodynamic effects of acute apelin administration are maintained in this patient population. In both patients and controls, intrabrachial apelin infusion increased FBF whist systemic intravenous infusion increased cardiac output and reduced peripheral vascular resistance. Heart rate increased transiently in controls but not patients. In both groups there was a small reduction in blood pressure but this appeared to be more sustained in the patient group.

Patients with advanced chronic heart failure exhibit an apparent down regulation of the apelin system that parallels findings in preclinical models and includes reduced cardiac expression of the apelin receptor [Földes *et al* 2003; Chong *et al* 2006]. Importantly, these patients were maintained on current optimal pharmacological therapy, suggesting that the effects of apelin were additive to established heart failure treatments. In particular, all but one of the patients were receiving treatment with an ACE inhibitor or angiotensin receptor blocker therapy. Recent reports of direct interactions between the renin-angiotensin and apelin systems have raised the possibility that some of the actions of apelin may be mediated through antagonism of angiotensin II [Iwanaga *et al* 2006; Chun *et al* 2008]. However the findings in this thesis imply a role for apelin that is independent of angiotensin II signalling pathways, and suggest potential for pharmacological synergism through combined apelin receptor agonism and renin-angiotensin system inhibition.

7.5 COMPARISON OF CARDIOVASCULAR RESPONSES TO DIFFERENT APELIN PEPTIDES

The identity of the principal endogenous ligand for the apelin receptor *in vivo* remains uncertain. In this thesis I compared cardiovascular responses to apelin-36, the full-length mature peptide, with (Pyr^{1})apelin-13, recently shown to be the predominant isoform in human plasma and cardiac tissue. In the forearm vascular bed there was no difference in vasodilatation induced by the two apelin isoforms over a range of subsystemic doses. This is consistent with recent *in vitro* data showing that the same two peptides caused vasodilatation in human mammary artery with comparable potency [Maguire *et al* 2009]. Similarly, the magnitude of systemic haemodynamic responses to the two isoforms did not differ over the range of doses used in this study. This contrasts with the finding in rodents that shorter apelin fragments induce greater depressor effects than apelin-36 [Tatemoto *et al* 2001], suggesting possible interspecies differences in the responses to different apelin fragments. However, it may also reflect the greater interval between dosing and haemodynamic measurements in these studies as the differing responses in rodents were most apparent within the first 3 minutes after administration.

I have demonstrated that both the direct vascular and systemic haemodynamic effects of apelin-36 have a more prolonged offset than the shorter isoform with vasodilatation persisting for at least 42 minutes after drug cessation. These findings mirror *in vitro* data showing rapid dissociation from the apelin receptor and transient

intracellular responses with (Pyr[1])apelin-13 compared with much slower dissociation and prolonged receptor activation with apelin-36 [Hosoya *et al* 2000; Masri *et al* 2006]. Such pharmacodynamic differences might potentially underpin distinct physiological roles for the different apelin isoforms *in vivo*.

7.6 FUTURE DIRECTIONS

7.6.1 APELIN ANTAGONISM

Recently, Macaluso *et al* provided a report of the first small-molecule apelin antagonist, cyclo(1-6)CRPRLC-KH-cyclo(9-14)CRPRLC, demonstrating competitive inhibition of apelin-induced receptor internalisation and inhibition of cAMP production in cell lines transfected with the human apelin receptor [Macaluso *et al* 2011]. Pending confirmation of its efficacy, this agent could be used to characterise the cardiovascular actions of endogenous apelin both in preclinical models and clinical studies.

In preclinical models, the vascular effects of exogenous apelin have been studied directly through wire myography [Salcedo *et al* 2007] and indirectly, *in vivo*, through changes in systemic blood pressure [Lee *et al* 2000, Tatemoto *et al* 2001], left ventricular filling pressures [Ashley *et al* 2005] and mean capillary filling pressure [Cheng *et al* 2003]. Additionally, the direct inotropic actions of exogenous apelin have been demonstrated *in vitro*, in single cardiomyocytes [Farkasfalvi *et al* 2007], paced atrial strips [Dai *et al* 2006; Maguire *et al* 2009] and Langendorff hearts [Szokodi *et al* 2002] and *in vivo,* by pressure-volume haemodynamics [Ashley *et al*

2005]. By combining these techniques with a selective apelin receptor antagonist, the role of endogenous apelin signalling in the regulation of cardiac contractility, and its contribution to the basal vascular tone could be established.

Findings from murine models with deletion of the apelin or apelin receptor gene indicate a role for endogenous apelin signalling in preserving cardiac function during cardiovascular stress [Kuba *et al* 2007]. Correspondingly, the apelin system appears to be up regulated in the early stages of heart failure [Iwanaga *et al* 2006]. However, both expression of the apelin receptor and plasma apelin concentrations decline in advanced symptomatic disease [Iwanaga *et al* 2006; Jia *et al* 2006]. The pathophysiologic significance of changes in expression of the apelin pathway could be explored by examining the effects of apelin antagonism on cardiac function and systemic haemodynamics at different disease stages in animal models with predictable progression to heart failure e.g. the Dahl salt-sensitive rat, correlating observed differences with changes in expression of apelin peptides and the apelin receptor.

This thesis has characterised the acute cardiovascular effects of exogenous apelin in man. Owing to the lack of a validated apelin receptor antagonist at the time of the studies, I was unable to explore the role of endogenous apelin signalling in man. However, the techniques utilised in these studies could be readily applied to the study of apelin antagonism. Forearm venous occlusion plethysmography during continuous intrabrachial infusion of a putative apelin receptor antagonist or saline placebo with simultaneous graded infusions of apelin and control vasodilators could

be used to confirm its selective antagonism of the apelin receptor in man. Thereafter, the effects of the antagonist on forearm and coronary blood flow using the methodology described in Chapter 3 would reveal the contribution of endogenous apelin to the peripheral and coronary basal vascular tone in man. Intravenous infusions of an apelin antagonist combined with bioimpedance cardiography or pulmonary artery catheterisation could then be employed to establish the composite effects of endogenous apelin signalling on systemic haemodynamics. In these studies, simultaneous forearm venous occlusion plethysmography with locally active intrabrachial infusions of apelin receptor agonists would confirm the efficacy of the chosen systemic antagonist doses. Finally, potential alterations in endogenous apelin signalling in the setting of chronic heart failure, could be evaluated by determining the local vascular and systemic haemodynamic effects of apelin antagonism in patients with chronic heart failure using the methodology described in Chapter 6.

7.6.2 THERAPEUTIC POTENTIAL OF CHRONIC APELIN RECEPTOR AGONISM

The cardiovascular effects of acute apelin administration in rodents are now relatively well characterised but the impact of chronic administration requires further study. One of the most encouraging findings to date with apelin is the enhancement of cardiac performance with chronic administration that occurred without inducing left ventricular hypertrophy [Ashley et al 2005]. Further studies are required to confirm this finding over longer treatment periods and to clarify the effects, if any, of chronic apelin receptor agonism on cardiac expression of apelin receptors. Additionally the effects of chronic apelin administration in animal models of heart failure should be examined to determine the ability of apelin receptor agonism to

prevent or delay decline in cardiac function as well as to ameliorate the effects of established heart failure.

In this thesis, I have assessed only the acute effects of apelin administration. The consequences of chronic apelin administration or agonism in patients with chronic heart failure cannot be inferred from these studies. Chronic dosing is not currently practicable or deliverable given the need for parenteral administration of apelin peptide. This problem could be addressed by the development of long-acting orally active apelin agonists or selective inhibitors of apelin degradation. However the enzymatic pathways responsible for inactivation of apelin peptides are presently unknown and, although the first non-peptidic apelin agonist, E339-3D6, has recently been described [Iturrioz *et al* 2010], to date, there are no reports of orally active apelin agonists. As an interim step, the effects of prolonged apelin infusion over a period of hours on cardiac contractility, systemic haemodynamics and fluid balance could be characterised. Confirmation of sustained arterial and venous dilatation, positive inotropy and diuretic effects over this time period would provide a rationale for a clinical trial to assess the therapeutic efficacy of intravenous apelin infusion in patients with acute decompensated heart failure. In addition to standard therapy with oxygen, diuretic and nitrates, patients presenting to the emergency department with acute pulmonary oedema could be randomised to a continuous 6-hour infusion of apelin or matched placebo. Appropriate endpoints could include improvement in arterial acidosis, breathlessness (visual analogue scale), neurohormonal markers, glomerulofiltration rate, intubation rate, length of hospital stay and in-hospital mortality.

7.6.3 DIRECT CARDIAC EFFECTS OF APELIN *IN VIVO*

Given the vasoactive actions of apelin described in this thesis, an assessment of its direct myocardial effects *in vivo* cannot be achieved using standard, load-dependent measures of contractility such as dP/dtmax or ejection fraction. To overcome this problem, the effects of apelin on load-independent indices of myocardial contractility could be studied using pressure-volume haemodynamics with the aid of a conductance catheter system e.g. CD Leycom CFL-512. This dedicated catheter system has a micromanometer to measure intraventricular pressures and sequential electrical impedance to measure ventricular volume. During intracoronary infusions of apelin or matched saline placebo, pressure changes within the left ventricle could be combined with volume data to form real-time pressure-volume loops over successive cardiac cycles, both at baseline and following manipulation of preload [Wittstein *et al* 2001]. In addition to standard measures of cardiac contractility and loading such as cardiac output, dP/dtmax, end-diastolic and end-systolic volumes and ejection fraction, this approach would permit measurement of end-systolic elastance (dP/dtmax/end-diastolic volume index) and end-systolic pressure-volume ratio as load-independent indices of systolic function [Kass and Maughan 1988]. Thus, effects of load and intrinsic contractility can be simultaneously and independently assessed. The same methodology combined with a validated apelin receptor antagonist could then be usd to determine the effects of endogenous apelin on myocardial contractility.

7.6.4 APELIN AND VASCULAR DISEASE

This thesis has demonstrated an increase in coronary blood flow following intracoronary apelin injection. However, the coronary vasomotor actions of apelin require further study. Graded infusions of apelin in place of two single intracoronary injections would permit more detailed characterisation of the time course and dose-dependence of its effects. In addition, measurement of coronary artery cross-sectional area with intravascular ultrasound might allow a more accurate assessment of epicardial vessel size than is achievable with QCA. Apelin potently constricts human atherosclerotic coronary artery. Paradoxical vasoconstriction of atherosclerotic coronary arteries has been described *in vivo* with other endothelium-dependent vasodilators such as acetylcholine [Ludmer *et al* 1986] and this would have implications for the effects of apelin in patients with coronary artery disease. The direct coronary effects of apelin in patients with significant coronary artery disease and other conditions associated with endothelial dysfunction will therefore need to be examined.

Apelin appears to have beneficial effects on vascular health. In diabetic mice, it increases vascular nitric oxide generation and reverses endothelial dysfunction [Zhong *et al* 2007]. The therapeutic potential of apelin receptor agonism in human vascular disease has yet to be explored. In this thesis I have demonstrated that apelin causes nitric oxide-mediated vasodilatation *in vivo* in forearm resistance vessels of healthy subjects but it is not yet known whether it reverses the endothelial dysfunction seen in pathophysiological states such as diabetes mellitus and hypercholesterolaemia. This question could be addressed by comparing vasodilator

responses to endothelium-dependent and -independent vasodilators in the forearm vascular bed of patients with these conditions following local intrabrachial infusion of apelin or saline placebo.

Preclinical models and preliminary clinical data indicate that diminished apelin signalling may contribute to the pathogenesis of pulmonary hypertension [Alastalo *et al* 2011; Chandra *et al* 2011]. Furthermore, in line with findings in the systemic circulation, apelin causes nitric oxide-mediated vasorelaxation in pulmonary artery *in vitro* [Huang *et* al 2011] and a modest reduction in pulmonary artery pressure *in vivo* [Feng *et al* 2010]. The vasomotor effects of apelin in the pulmonary circulation have not been studied in man. This could be achieved by measurement of pulmonary haemodynamic indices with a pulmonary artery catheter during systemic intravenous infusions of apelin and matched saline placebo. Such a study would permit further examination of the effects of apelin on cardiac output and central filling pressures as well as pulmonary vascular resistance, the latter calculated by dividing the transpulmonary gradient by the cardiac output. After establishing the pulmonary haemodynamic effects of apelin in healthy volunteers, responses to apelin could be assessed in patients with idiopathic pulmonary arterial hypertension and pulmonary hypertension associated with left heart disease and compared with age- and sex-matched controls.

7.6.5 INTERACTION OF THE APELIN AND RENIN-ANGIOTENSIN SYSTEMS

The angiotensin II type 1 and apelin receptors exhibit close sequence homology and markedly similar patterns of tissue expression, yet apelin mediates opposing actions

to angiotensin II on vascular tone, blood pressure and fluid homeostasis [Ashley *et al* 2006]. These findings suggest a counter-regulatory role for apelin in relation to the renin-angiotensin system, and recent preclinical data support this hypothesis. Apelin antagonises the proatherogenic effects of angiotensin II, and attenuates angiotensin II-mediated constriction of veins and arteries in rodent models [Guzru *et al* 2006; Chun *et al* 2008]. In contrast, mice with knockout of the apelin receptor gene exhibit an exaggerated pressor response to exogenous angiotensin II [Ishida *et al* 2004]. Given that apelin selectively inhibits angiotensin II-mediated transcription of multiple gene targets [Chun *et al* 2008], this regulation may occur, at least in part, through direct antagonism of angiotensin II signalling pathways.

As shown in this thesis, local apelin infusion does not alter venous tone in the dorsal hand vein. Therefore, to determine whether apelin directly and selectively inhibits the acute vasomotor actions of angiotensin II *in vivo* in man, venoconstrictor responses to local intravenous infusions of angiotensin II and the control vasoconstricitor norepinephrine could be compared using the dorsal hand vein technique during co-infusion of apelin or saline placebo. To further explore the direct effects of apelin on vascular responses to angiotensin II, constrictor responses to intrabrachial angiotensin II and the control vasoconstricitor norepinephrine could be compared in the forearm circulation in the presence and absence of apelin or SNP (titrated to achieve equivalent baseline blood flow). By employing subsytemic doses of drugs in these studies, potentially confounding effects of angiotensin II and apelin on renal, endocrine and systemic haemodynamic factors will be avoided. In addition,

the control vasoconstrictor and vasodilator infusions will account for the non-specific vasomotor effects.

There is also increasing evidence for direct counter-regulation of the apelin system by angiotensin II. In rats, infusion of angiotensin II for 24 hours, even at subpressor doses, reduces cardiac apelin expression, an effect abolished by concurrent AT_1 receptor blockade [Iwanaga *et al* 2006]. Similarly, in a rodent model of heart failure, several pharmacological treatments retard progression to chronic heart failure, but only AT_1 receptor antagonism prevents down regulation of apelin and apelin receptor expression [Iwanaga *et* al 2006]. While basal blood pressure is normal in mice with selective knockout of the apelin receptor gene, it is elevated in mice with combined apelin and AT_1 receptor knockout implying that, in the absence of angiotensin II activity, apelin contributes to basal vascular tone [Ishida *et al* 2004]. Finally angiotensin II has recently been shown to induce heterodimerisation of the apelin and AT_1 receptors [Chun *et al* 2008]**,** thereby providing a plausible mechanism for direct inhibition of apelin signalling by angiotensin II.

To date there are no reports on the interactions between the apelin and renin-angiotensin systems in man. Sodium depletion is a well-validated and physiologically relevant method of renin-angiotensin system stimulation and can be achieved through a low salt diet as previously described. This could be used as a model to study changes in plasma apelin concentrations and the direct vascular and systemic haemodynamic responses to exogenous apelin during sustained up regulation of endogenous angiotensin II production. Whilst this provides a valid

approach to assessing neurohumoral activation in healthy volunteers it must be acknowledged that sodium depletion will lead to alterations in other neurohormonal systems, such as the vasopressin system. Therefore, to establish whether the *in vivo* vasomotor effects of apelin in man are directly and selectively inhibited by concurrent AT_1 receptor activation, FBF responses to intrabrachial infusion of apelin or the control endothelium-dependent vasodilator, acetylcholine, could be compared in the presence of angiotensin II or the control vasoconstrictor, norepinephrine. Similarly systemic haemodynamic responses to apelin could be compared during co-infusion of angiotensin II at a subpressor dose such as 0.5 mg/kg/min [Ljungmanet *et al* 1983; Miller *et al* 1999] or saline placebo. Finally, following the development and validation of suitable antagonists of the apelin receptor these same protocols could be employed to assess the effects of differential states of renin-angiotensin system activation on endogenous apelin activity.

7.7 CONCLUDING REMARKS

Fourteen years since the pairing of the apelin receptor with its endogenous ligand, the field of apelin biology is, in many respects, still in its infancy. Whilst increasing data suggest an important role for the apelin system in cardiovascular physiology and pathophysiology, many fundamental aspects of the system have yet to be established. In particular, further work is required to elucidate the regulation of gene transcription and the degradation pathway of apelin peptides; to reconcile phenotypic differences between receptor and ligand knockout models; to clarify intracellular signalling mechanisms; and to develop new pharmacological tools such as selective antagonists

and long-acting agonists to explore the physiological relevance of the apelin signalling. Advances in these areas will serve to inform and complement studies such as those described and others designed to clarify the physiological role and therapeutic potential of apelin signalling within the cardiovascular system.

REFERENCES

REFERENCES

Aellig WH. A new technique for recording compliance of human hand veins. *Br J Clin Pharmacol* 1981;**11**:237-243.

Aellig WH. Clinical pharmacology, physiology and pathophysiology of superficial veins-1. *Br J Clin Pharmacol* 1994a;**38**:181-196.

Aellig WH. Clinical pharmacology, physiology and pathophysiology of superficial veins-2. *Br J Clin Pharmacol* 1994b;**38**:289-305.

Affolter JT, McKee SP, Helmy A *et al*. Intra-arterial vasopressin in the human forearm: pharmacodynamics and the role of nitric oxide. *Clin Pharmacol Ther* 2003;**74**:9-16.

Alastalo TP, Li M, Perez Vde J *et al*. Disruption of PPARγ/β-catenin-mediated regulation of apelin impairs BMP-induced mouse and human pulmonary arterial EC survival. *J Clin Invest* 2011;**121**:3735-3746.

Appel PL, Kram HB, Mackabee J *et al*. Comparison of measurements of cardiac output by bioimpedance and thermodilution in severely ill surgical patients. *Crit Care Med* 1986;**14**:933-935.

Ashley EA, Powers J, Chen M *et al*. The endogenous peptide apelin potently improves cardiac contractility and reduces cardiac loading in vivo. *Cardiovasc Res* 2005;**65**:73-82.

Ashley E, Chun HJ, Quertermous T. Opposing cardiovascular roles for the angiotensin and apelin signaling pathways. *J Mol Cell Cardiol* 2006;**41**:778-781.

Atluri P, Morine KJ, Liao GP *et al*. Ischemic heart failure enhances endogenous myocardial apelin and APJ receptor expression. *Cell Mol Biol Lett* 2007;**12**:127-138.

Azizi M, Iturrioz X, Blanchard A *et al*. Reciprocal regulation of plasma apelin and vasopressin by osmotic stimuli. *J Am Soc Nephrol* 2008;**19**:1015-1024.

Barnes G, **Japp AG,** Newby DE. Translational promise of the apelin-APJ system**.** *Heart* 2010;**96**:1011-1016.

Benjamin N, Calver A, Collier J, Robinson B, Vallance P, Webb D. Measuring forearm blood flow and interpreting the responses to drugs and mediators. *Hypertension* 1995;**25**:918-923.

Bernstein DP. A new stroke volume equation for thoracic electrical bioimpedance: theory and rationale. *Crit Care Med* 1986;**14**:904-909.

Berry MF, Pirolli TJ, Jayasankar V *et al*. Apelin has in vivo inotropic effects on normal and failing hearts. *Circulation* 2004;**110**:II187-193.

Blows LJ, Redwood SR. The pressure wire in practice. *Heart* 2007;**93**:419-422.

Boucher J, Masri B, Daviaud D *et al*. Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology* 2005;**146**:1764-1771.

Calver A, Collier J, Vallance P. Inhibition and stimulation of nitric oxide synthesis in the human forearm arterial bed of patients with insulin-dependent diabetes. *J Clin Invest* 1992;**90**:2548-2554.

Campia U, Choucair WK, Bryant MB *et al*. Role of cyclooxygenase products in the regulation of vascular tone and in the endothelial vasodilator function of normal, hypertensive, and hypercholesterolemic humans. *Am J Cardiol* 2002;**89**:286-290.

Cayabyab M, Hinuma S, Farzan M *et* al. Apelin, the natural ligand of the orphan seven-transmembrane receptor APJ, inhibits human immunodeficiency virus type 1 entry. *J Virol* 2000;**74**:11972-11976.

Chandra SM, Razavi H, Kim J *et al*. Disruption of the apelin-APJ system worsens hypoxia-induced pulmonary hypertension. *Arterioscler Thromb Vasc Biol* 2011; **31**:814-820.

Charles CJ, Rademaker MT, Richards AM. Apelin-13 induces a biphasic haemodynamic response and hormonal activation in normal conscious sheep. *J Endocrinol* 2006;**189**:701-710.

Charo DN, Ho M, Fajardo G *et al*. Endogenous regulation of cardiovascular function by apelin-APJ. *Am J Physiol Heart Circ Physiol* 2009;**297**:H1904-1913.

Chen MM, Ashley EA, Deng DX *et al*. Novel role for the potent endogenous inotrope apelin in human cardiac dysfunction. *Circulation* 2003;**108**:1432-1439.

Cheng X, Cheng XS, Pang CC. Venous dilator effect of apelin, an endogenous peptide ligand for the orphan APJ receptor, in conscious rats. *Eur J Pharmacol* 2003;**470**:171-175.

Choe H, Farzan M, Konkel M *et al*. The orphan seven-transmembrane receptor APJ supports the entry of primary T-cell-line-tropic and dualtropic human immunodeficiency virus type 1. *J Virol* 1998;**72**:6113-6118.

Choe W, Albright A, Sulcove J *et al*. Functional expression of the seven-transmembrane HIV-1 co-receptor APJ in neural cells. *J Neurovirol* 2000;**6** (Suppl1):S61-69.

Chong KS, Gardner RS, Morton JJ, Ashley EA, McDonagh TA. Plasma concentrations of the novel peptide apelin are decreased in patients with chronic heart failure. *Eur J Heart Fail* 2006;**8**:355-360.

Chowienczyk PJ, Cockcroft JR, Ritter JM. Blood flow responses to intra-arterial acetylcholine in man: effects of basal flow and conduit vessel length. *Clin Sci (Lond)* 1994;**87**:45-51.

Chun HJ, Ali ZA, Kojima Y *et al*. Apelin signaling antagonizes Ang II effects in mouse models of atherosclerosis. *J Clin Invest* 2008;**118**:3343-3354.

CIBIS-II Investigators. The Cardiac Insufficiency Bisoprolol Study II (CIBIS-II): a randomised trial. *Lancet* 1999;**353**:9-13.

Cleland JG, Daubert JC, Erdmann E *et al*. The effect of cardiac resynchronization on morbidity and mortality in heart failure. *N Engl J Med* 2005;**352**:1539-1549.

Codognotto M, Piccoli A, Zaninotto M *et al*. Evidence for decreased circulating apelin beyond heart involvement in uremic cardiomyopathy. *Am J Nephrol* 2007;**27**:1-6.

Corre S, Galibert MD. [USF as a key regulatory element of gene expression]. *Med Sci (Paris)* 2006;**22**:62-67.

Cox CM, D'Agostino SL, Miller MK, Heimark RL, Krieg PA. Apelin, the ligand for the endothelial G-protein-coupled receptor, APJ, is a potent angiogenic factor required for normal vascular development of the frog embryo. *Dev Biol* 2006;**296**:177-189.

Cruden NL, Witherow FN, Webb DJ, Fox KA, Newby DE. Bradykinin contributes to the systemic haemodynamic effects of chronic angiotensin-converting enzyme inhibition in patients with heart failure. *Arterioscler Thromb Vasc Biol* 2004;**24**:1043-1048.

Dai T, Ramirez-Correa G, Gao WD. Apelin increases contractility in failing cardiac muscle. *Eur J Pharmacol* 2006;**553**:222-228.

De Falco M, De Luca L, Onori N *et al*. Apelin expression in normal human tissues. *In Vivo* 2002;**16**:333-336.

De Feyter PJ, Serruys PW, Davies MJ *et al*. Quantitative coronary angiography to measure progression and regression of coronary atherosclerosis. *Circulation* 1991;**84**:412-423.

De Mota N, Lenkei Z, Llorens-Cortes C. Cloning, pharmacological characterization and brain distribution of the rat apelin receptor. *Neuroendocrinology* 2000;**72**:400-407.

De Mota N, Reaux-Le Goazigo A, El Messari S *et al*. Apelin, a potent diuretic neuropeptide counteracting vasopressin actions through inhibition of vasopressin neuron activity and vasopressin release. *Proc Natl Acad Sci USA* 2004;**101:** 10464-10469.

Doucette JW, Corl PD, Payne HM *et al*. Validation of a Doppler guide wire for intravascular measurement of coronary artery flow velocity. *Circulation* 1992;**85**:1899-1911.

Dray C, Knauf C, Daviaud D *et al*. Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. *Cell Metab* 2008;**8**:437-445.

Dray C, Debard C, Jager J *et al*. Apelin and APJ regulation in adipose tissue and skeletal muscle of type 2 diabetic mice and humans. *Am J Physiol Endocrinol Metab* 2010;**298**:E1161-1169.

Duckett SG, Ginks M, Shetty Ak *et al*. Invasive acute hemodynamic response to guide left ventricular lead implantation predicts chronic remodeling in patients undergoing cardiac resynchronization therapy. *J Am Coll Cardiol* 2011;**58**:1128-1136.

Eggena P, Zhu JH, Clegg K, Barrett JD. Nuclear angiotensin receptors induce transcription of renin and angiotensinogen mRNA. *Hypertension* 1993;**22**:496-501.

El Messari S, Iturrioz X, Fassot C *et al*. Functional dissociation of apelin receptor signaling and endocytosis: implications for the effects of apelin on arterial blood pressure. *J Neurochem* 2004;**90**:1290-1301.

Eyries M, Siegfried G, Ciumas M *et al*. Hypoxia-induced apelin expression regulates endothelial cell proliferation and regenerative angiogenesis. *Circ Res* 2008;**15**;103:432-440.

Fan X, Zhou N, Zhang X *et al.* Structural and functional study of the apelin-13 peptide, an endogenous ligand of the HIV-1 coreceptor, APJ. *Biochemistry* 2003;**42**:10163-10168.

Farkasfalvi K, Stagg MA, Coppen SR *et al*. Direct effects of apelin on cardiomyocyte contractility and electrophysiology. *Biochem Biophys Res Commun* 2007;**357**:889-895.

Feng JH, Li WM, Wu XP *et al*. Hemodynamic effect of apelin in a canine model of acute pulmonary thromboembolism. *Peptides* 2010;**31**:1772-1778.

Földes G, Horkay F, Szokodi I *et al*. Circulating and cardiac levels of apelin, the novel ligand of the orphan receptor APJ, in patients with heart failure. *Biochem Biophys Res Commun* 2003;**308**:480-485.

Foord SM, Bonner TI, Neubig RR *et al*. International Union of Pharmacology. XLVI. G protein-coupled receptor list. *Pharmacol Rev* 2005;**57**:279-288.

Francia P, Salvati A, Balla C *et al*. Cardiac resynchronization therapy increases plasma levels of the endogenous inotrope apelin. *Eur J Heart Fail* 2007;**9**:306-309.

Geiger K, Muendlein A, Stark N *et al*. Hypoxia induces apelin expression in human adipocytes. *Horm Metab Res* 2011;**43**:380-385.

Ginks MR, Sciaraffia E, Karlsson A *et al*. Relationship between intracardiac impedance and left ventricular contractility in patients undergoing cardiac resynchronization therapy. *Europace* 2011;**13**:984-991.

Goetze JP, Rehfeld JF, Carlsen J *et al*. Apelin: a new plasma marker of cardiopulmonary disease. *Regul Pept* 2006;**133**:134-138.

Gudmundsdóttir IJ, Megson IL, Kell JS *et al*. Direct vascular effects of protease-activated receptor type 1 agonism *in vivo* in humans. *Circulation* 2006;**114**:1625-1632.

Gurzu B, Petrescu BC, Costuleanu M *et al*. Interactions between apelin and angiotensin II on rat portal vein. *J Renin Angiotensin Aldosterone Syst* 2006;**7**: 212-216.

Habata Y, Fujii R, Hosoya M *et al*. Apelin, the natural ligand of the orphan receptor APJ, is abundantly secreted in the colostrum. *Biochim Biophys Acta* 1999;**1452**: 25-35.

Haber E, Koerner T, Page LB, Kliman B, Purnode A. Application of a radioimmunoassay for angiotensin I to the physiologic measurements of plasma renin activity in normal human subjects. *J Clinical Endocrinol Metab* 1969;**29**:1349-1355.

Hamming I, Cooper ME, Haagmans BL *et al*. The emerging role of ACE2 in physiology and disease. *J Pathol* 2007;**212**:1-11.

Hashimoto T, Kihara M, Ishida J *et al*. Apelin stimulates myosin light chain phosphorylation in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2006;**26**:1267-1272.

Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. *Cardiovasc Res* 2004;**61**:448-460.

Haynes WG, Noon J, Walker BR, Webb DJ. L-NMMA increases blood pressure in humans. *Lancet*. 1993;**342**:931-932.

Haynes WG, Hand MF, Johnstone HA, Padfield PL, Webb DJ. Direct and sympathetically mediated venoconstriction in essential hypertension. Enhanced responses to endothelin-1. *J Clin Invest* 1994;**94**:1359-1364.

Haynes WG, Strachan FE, Webb DJ. Endothelin ETA and ETB receptors cause vasoconstriction of human resistance and capacitance vessels *in vivo*. *Circulation*. 1995;**92**:357-363.

Heavey DJ, Barrow SE, Hickling NE, Ritter JM. Aspirin causes short-lived inhibition of bradykinin-stimulated prostacyclin production in man. *Nature* 1985;**318**:186-188.

Higuchi K, Masaki T, Gotoh K *et al*. Apelin, an APJ receptor ligand, regulates body adiposity and favors the messenger ribonucleic acid expression of uncoupling proteins in mice. *Endocrinology* 2007;**148**:2690-2697.

Hirooka Y, Egashira K, Imaizumi T *et al*. Effect of L-arginine on acetylcholine-induced endothelium-dependent vasodilation differs between the coronary and forearm vasculatures in humans. *J Am Coll Cardiol* 1994;**24**:948-955.

Honing M, Smits P, Morrison P, Rabelink T. Bradykinin-induced vasodilation of human forearm resistance vessels is primarily mediated by endothelium-dependent hyperpolarization. *Hypertension* 2000;**35**:1314-1318.

Hood WP Jr, Amende I, Simon R, Lichtlen PR. The effects of intracoronary nitroglycerin on left ventricular systolic and diastolic function in man. *Circulation* 1980;**61**:1098-1104.

Hosoya M, Kawamata Y, Fukusumi S *et al*. Molecular and functional characteristics of APJ. Tissue distribution of mRNA and interaction with the endogenous ligand apelin. *J Biol Chem* 2000;**275**:21061-21067.

Huang P, Fan XF, Pan LX *et al*. Effect of apelin on vasodilatation of isolated pulmonary arteries in rats is concerned with the nitric oxide pathway. *Zhongguo Ying Yong Sheng Li Xue Za Zhi* 2011;**27**:1-5.

Hus-Citharel A, Bouby N, Frugière A, Bodineau L, Gasc JM, Llorens-Cortes C. Effect of apelin on glomerular hemodynamic function in the rat kidney. *Kidney Int* 2008;**74**:486-494.

Ishida J, Hashimoto T, Hashimoto Y *et al*. Regulatory roles for APJ, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure in vivo. *J Biol Chem* 2004;**279**:26274-26279.

Iturrioz X, Alvear-Perez R, De Mota N *et al*. Identification and pharmacological properties of E339-3D6, the first nonpeptidic apelin receptor agonist. *FASEB J* 2010;**24**:1506-1517.

Iwanaga Y, Kihara Y, Takenaka H, Kita T. Down-regulation of cardiac apelin system in hypertrophied and failing hearts: Possible role of angiotensin II-angiotensin type1 receptor system. *J Mol Cell Cardiol* 2006;**41**:798-806.

Japp AG, Cruden NL, Amer DA *et al*. Vascular effects of apelin in vivo in man. *J Am Coll Cardiol* 2008;**52**:908-913.

Japp AG, Newby DE. The apelin-APJ system in heart failure: pathophysiologic relevance and therapeutic potential. *Biochem Pharmaco*. 2008;**75**:1882-1892.

Japp AG, Cruden NL, Barnes G *et al.* Acute cardiovascular effects of apelin in humans: potential role in patients with chronic heart failure. *Circulation* 2010;**121**:1818-1827.

Jászberényi M, Bujdosó E, Telegdy G. Behavioral, neuroendocrine and thermoregulatory actions of apelin-13. *Neuroscience* 2004;**129**:811-816.

Jia YX, Pan CS, Zhang J *et al*. Apelin protects myocardial injury induced by isoproterenol in rats. *Regul Pept* 2006;**133**:147-154.

Jia YX, Lu ZF, Zhang J *et al*. Apelin activates l-arginine/nitric oxide synthase/nitric oxide pathway in rat aortas. *Peptides* 2007;**28**:2023-2029.

Karmazyn M, Gan XT, Humphreys RA, Yoshida H, Kusumoto K. The myocardial Na(+)-H(+) exchange: structure, regulation, and its role in heart disease. *Circ Res* 1999;**85**:777-786.

Kasai A, Shintani N, Oda M *et al*. Apelin is a novel angiogenic factor in retinal endothelial cells. *Biochem Biophys Res Commun* 2004;**325**:395-400.

Kasai A, Shintani N, Kato H *et al*. Retardation of retinal vascular development in apelin-deficient mice. *Arterioscler Thromb Vasc Biol* 2008;**28**:1717-1722.

Kass DA, Maughan WL. From 'Emax' to pressure-volume relations: a broader view. *Circulation* 1988;**77**:1203-1212.

Katugampola SD, Maguire JJ, Matthewson SR, Davenport AP. [(125)I]-(Pyr(1))Apelin-13 is a novel radioligand for localizing the APJ orphan receptor in human and rat tissues with evidence for a vasoconstrictor role in man. *Br J Pharmacol* 2001;**132**:1255-1260.

Kawamata Y, Habata Y, Fukusumi S *et al*. Molecular properties of apelin: tissue distribution and receptor binding. *Biochim Biophys Acta* 2001;**1538**:162-171.

Kentish JC. A role for the sarcolemmal Na(+)/H(+) exchanger in the slow force response to myocardial stretch. *Circ Res* 1999;**85**:658-660.

Kidoya H, Ueno M, Yamada Y *et al*. Spatial and temporal role of the apelin/APJ system in the caliber size regulation of blood vessels during angiogenesis. *EMBO J* 2008;**27**:522-534.

Kidoya H, Naito H, Takakura N. Apelin induces enlarged and nonleaky blood vessels for functional recovery from ischemia. *Blood* 2010;**115**:3166-3174.

Kleinz MJ and Davenport AP. Immunocytochemical localisation of the endogenous vasoactive peptide apelin to human vascular and endocardial endothelial cells. *Regul Pept* 2004;**118**:119-125.

Kleinz MJ and Davenport AP. Emerging roles of apelin in biology and medicine. *Pharmacol Ther* 2005;**107**:198-211.

Kleinz MJ, Skepper JN, Davenport AP. Immunocytochemical localisation of the apelin receptor, APJ, to human cardiomyocytes, vascular smooth muscle and endothelial cells. *Regul Pept* 2005;**126**:233-240.

Kuba K, Zhang L, Imai Y *et al*. Impaired heart contractility in apelin gene-deficient mice associated with aging and pressure overload. *Circ Res* 2007;**101**:e32-42.

Kunduzova O, Alet N, Delesque-Touchard N *et al*. Apelin/APJ signaling system: a potential link between adipose tissue and endothelial angiogenic processes. *FASEB J* 2008;**22**:4146-4153.

Lambrecht NW, Yakubov I, Zer C, Sachs G. Transcriptomes of purified gastric ECL and parietal cells: identification of a novel pathway regulating acid secretion. *Physiol Genomics* 2006;**25**:153-165.

Lee DK, Cheng R, Nguyen T *et al*. Characterization of apelin, the ligand for the APJ receptor. *J Neurochem* 2000;**74**:34-41.

Lee DK, Lanca AJ, Cheng R *et al*. Agonist-independent nuclear localization of the Apelin, angiotensin AT1, and bradykinin B2 receptors. *J Biol Chem* 2004;**279**: 7901-7908.

Lee DK, Saldivia VR, Nguyen T, Cheng R, George SR, O'Dowd BF. Modification of the terminal residue of apelin-13 antagonizes its hypotensive action. *Endocrinology* 2005;**146**:231-236.

Leslie SJ, Spratt JC, McKee SP *et al*. Direct comparison of selective endothelin A and non-selective endothelin A/B receptor blockade in chronic heart failure. *Heart* 2005;**91**:914-919.

Li L, Yang G, Li Q *et al*. Changes and relations of circulating visfatin, apelin, and resistin levels in normal, impaired glucose tolerance, and type 2 diabetic subjects. *Exp Clin Endocrinol Diabetes* 2006;**114**:544-548.

Ljungman S, Aurell M, Hartford M, Wikstrand J, Berglund G. Effects of subpressor doses of angiotensin II on renal hemodynamics in relation to blood pressure. *Hypertension* 1983;**5**:368-374.

Ludmer PL, Selwyn AP, Shook TL *et al*. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl J Med* 1986;**315**:1046-1051.

Macaluso NJ, Pitkin SL, Maguire JJ, Davenport AP, Glen RC. Discovery of a competitive apelin receptor (APJ) antagonist. *ChemMedChem* 2011;**6**:1017-1023.

Maguire JJ, Davenport AP. Regulation of vascular reactivity by established and emerging GPCRs. *Trends Pharmacol Sci* 2005;**26**:448-454.

Maguire JJ, Kleinz MJ, Pitkin SL, Davenport AP. [Pyr1]apelin-13 identified as the predominant apelin isoform in the human heart: vasoactive mechanisms and inotropic action in disease. *Hypertension* 2009;**54**:598-604.

Masri B, Lahlou H, Mazarguil H, Knibiehler B, Audigier Y. Apelin (65-77) activates extracellular signal-regulated kinases via a PTX-sensitive G protein. *Biochem Biophys Res Commun* 2002;**290**:539-545.

Masri B, Morin N, Cornu M, Knibiehler B, Audigier Y. Apelin (65-77) activates p70 S6 kinase and is mitogenic for umbilical endothelial cells. *FASEB J* 2004;**18**: 1909-1911.

Masri B, Morin N, Pedebernade L, Knibiehler B, Audigier Y. The apelin receptor is coupled to Gi1 or Gi2 protein and is differentially desensitized by apelin fragments. *J Biol Chem* 2006;**281**:18317-18326.

McMurray JJ, Ostergren J, Swedberg K *et al*. Effects of candesartan in patients with chronic heart failure and reduced left-ventricular systolic function taking angiotensin-converting-enzyme inhibitors: the CHARM-Added trial. *Lancet* 2003;**362**:767-771.

Medhurst AD, Jennings CA, Robbins MJ *et al*. Pharmacological and immuno-histochemical characterization of the APJ receptor and its endogenous ligand apelin. *J Neurochem* 2003;**84**:1162-1172.

Melgar-Lesmes P, Pauta M, Reichenbach V *et al*. Hypoxia and proinflammatory factors upregulate apelin receptor expression in human stellate cells and hepatocytes. *Gut* 2011;**60**:1404-1411.

MERIT-HF Study Group**.** Effect of metoprolol CR/XL in chronic heart failure: Metoprolol CR/XL Randomised Intervention Trial in Congestive Heart Failure (MERIT-HF). *Lancet* 1999;**353**:2001-2007.

Miettinen KH, Magga J, Vuolteenaho O *et al*. Utility of plasma apelin and other indices of cardiac dysfunction in the clinical assessment of patients with dilated cardiomyopathy. *Regul Pept* 2007;**140**:178-184.

Miller JA, Thai K, Scholey JW. Angiotensin II type 1 receptor gene polymorphism predicts response to losartan and angiotensin II. *Kidney Int* 1999;**56**:2173-2180.

Mills NL, Törnqvist H, Robinson SD *et al*. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. *Circulation* 2005;**112**:3930-3936.

Mosterd A, Hoes AW. Clinical epidemiology of heart failure. *Heart* 2007;**93**:1137-1146.

Negrao CE, Hamilton MA, Fonarow GC, Hage A, Moriguchi JD, Middlekauff HR. Impaired endothelium-mediated vasodilation is not the principal cause of vasoconstriction in heart failure. *Am J Physiol* 2000;**278**:H168-174.

Neves SR, Ram PT, Iyengar R. G protein pathways. *Science* 2002;**296**:1636-1639.

Newby DE, Wright RA, Ludlam CA *et al*. An *in vivo* model for the assessment of acute fibrinolytic capacity of the endothelium. *Thromb Haemost* 1997a;**78**:1242-1248.

Newby DE, Boon NA, Webb DJ. Comparison of forearm vasodilatation to substance P and acetylcholine: contribution of nitric oxide. *Clin Sci (Lond)* 1997b;**92**:133-138.

Newby DE, Sciberras DG, Mendel CM, Gertz BJ, Boon NA, Webb DJ. Intra-arterial substance P mediated vasodilatation in the human forearm: pharmacology, reproducibility and tolerability. *Br J Clin Pharmacol* 1997c;**43**:493-499.

Newby DE, Wright RA, Labinjoh C *et al*. Endothelial dysfunction, impaired endogenous fibrinolysis, and cigarette smoking: a mechanism for arterial thrombosis and myocardial infarction. *Circulation* 1999;**99**:1411-1415.

Newby DE. Intracoronary infusions and the assessment of coronary blood flow in clinical studies. *Heart* 2000;**84**;118-120.

Newby DE, McLeod AL, Uren NG *et al*. Impaired coronary tissue plasminogen activator release is associated with coronary atherosclerosis and cigarette smoking: direct link between endothelial dysfunction and atherothrombosis. *Circulation* 2001; **103**:1936-1941.

Noon JP, Walker BR, Hand MF *et al*. Impairment of forearm vasodilatation to acetylcholine in hypercholesterolemia is reversed by aspirin. *Cardiovasc Res* 1998;**38**:480-484.

Northridge DB, Findlay IN, Wilson J *et al*. Non-invasive determination of cardiac output by Doppler echocardiography and electrical bioimpedance. *Br Heart J* 1990; **63**:93-97.

O'Carroll AM, Don AL, Lolait SJ. APJ receptor mRNA expression in the rat hypothalamic paraventricular nucleus: regulation by stress and glucocorticoids. *J Neuroendocrinol* 2003;**15**:1095-1101.

O'Carroll AM, Lolait SJ. Regulation of rat APJ receptor messenger ribonucleic acid expression in magnocellular neurones of the paraventricular and supraoptic nuclei by osmotic stimuli. *J Neuroendocrinol* 2003;**15**:661-666.

O'Carroll AM, Lolait SJ, Howell GM. Transcriptional regulation of the rat apelin receptor gene: promoter cloning and identification of an Sp1 site necessary for promoter activity. *J Mol Endocrinol* 2006;**36**:221-235.

O'Dowd BF, Heiber M, Chan A *et al*. A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene* 1993;**136**:355-360.

Packer M, Coats AJ, Fowler MB *et al*. Effect of carvedilol on survival in severe chronic heart failure. *N Engl J Med* 2001;**344**:1651-1658.

Pang CC. Measurement of body venous tone. *J Pharmacol Toxicol Methods* 2000;**44**:341-60.

Pitkin SL, Maguire JJ, Bonner TI, Davenport AP. International Union of Basic and Clinical Pharmacology. LXXIV. Apelin receptor nomenclature, distribution, pharmacology, and function. *Pharmacol Rev* 2010a;**62**:331-342.

Pitkin SL, Maguire JJ, Kuc RE, Davenport AP. Modulation of the apelin/APJ system in heart failure and atherosclerosis in man. *Br J Pharmacol* 2010b;**160**:1785-1795.

Pugh BF, Tjian R. Transcription from a TATA-less promoter requires a multisubunit TFIID complex. *Genes Dev* 1991;**5**:1935-1945.

Rahimtoola SH. Left ventricular end-diastolic and filling pressures in assessment of ventricular function. *Chest* 1973;**63**:858-860.

Reaux A, De Mota N, Skultetyova I *et al*. Physiological role of a novel neuropeptide, apelin, and its receptor in the rat brain. *J Neurochem* 2001;**77**:1085-1096.

Reaux-Le Goazigo A, Morinville A, Burlet A, Llorens-Cortes C, Beaudet A. Dehydration-induced cross-regulation of apelin and vasopressin immunoreactivity levels in magnocellular hypothalamic neurons. *Endocrinology* 2004;**145**:4392-4400.

Roberts EM, Newson MJ, Pope GR, Landgraf R, Lolait SJ, O'Carroll AM. Abnormal fluid homeostasis in apelin receptor knockout mice. *J Endocrinol* 2009;**202**:453-462.

Ronkainen VP, Ronkainen JJ, Hanninen SL *et al*. Hypoxia inducible factor regulates the cardiac expression and secretion of apelin. *FASEB J* 2007;**21**:1821-1830.

Salandin V, Zussa C, Risica G *et al*. Comparison of cardiac output estimation by thoracic electrical bioimpedance, thermodilution, and Fick methods. *Crit Care Med* 1988;**16**:1157-1158.

Salcedo A, Garijo J, Monge L *et al*. Apelin effects in human splanchnic arteries. Role of nitric oxide and prostanoids. *Regul Pept* 2007;**114**:50-55.

Sarzani R, Forleo C, Pietrucci F *et al*. The 212A variant of the APJ receptor gene for the endogenous inotrope apelin is associated with slower heart failure progression in idiopathic dilated cardiomyopathy. *J Card Fail* 2007;**13**:521-529.

Schmitt M, Blackman DJ, Middleton GW, Cockcroft JR, Frenneaux MP. Assessment of venous capacitance. Radionuclide plethysmography: methodology and research applications. *Br J Clin Pharmacol* 2002;**54**:565-576.

Scott IC, Masri B, D'Amico LA *et al*. The g protein-coupled receptor agtrl1b regulates early development of myocardial progenitors. *Dev Cell* 2007;**12**:403-413.

Sheikh AY, Chun HJ, Glassford AJ *et al*. In vivo genetic profiling and cellular localization of apelin reveals a hypoxia-sensitive, endothelial-centered pathway activated in ischemic heart failure. *Am J Physiol Heart Circ Physiol* 2008;**294**: H88-98.

Simpkin JC, Yellon DM, Davidson SM, Lim SY, Wynne AM, Smith CC. Apelin-13 and apelin-36 exhibit direct cardioprotective activity against ischemiareperfusion injury. *Basic Res Cardiol* 2007;**102**:518-528.

Sörhede Winzell M, Magnusson C, Ahrén B. The apj receptor is expressed in pancreatic islets and its ligand, apelin, inhibits insulin secretion in mice. *Regul Pept* 2005;**131**:12-17.

Sorli SC, van den Berghe L, Masri B, Knibiehler B, Audigier Y. Therapeutic potential of interfering with apelin signalling. *Drug Discov Today* 2006;**11**:1100-1106.

Sorli SC, Le Gonidec S, Knibiehler B, Audigier Y. Apelin is a potent activator of tumour neoangiogenesis. *Oncogene* 2007;**26**:7692-7699.

Stroes ES, Koomans HA, de Bruin TW *et al*. Vascular function in the forearm of hypercholesterolaemic patients off and on lipid-lowering medication. *Lancet* 1995;**346**:467-471.

Stroes ES, Luscher TF, de Groot FG *et al*. Cyclosporin A increases nitric oxide activity in vivo. *Hypertension* 1997;**29**:570-575.

Sunter D, Hewson AK, Dickson SL. Intracerebroventricular injection of apelin-13 reduces food intake in the rat. *Neurosci Lett* 2003;**353**:1-4.

Szokodi I, Tavi P, Foldes G *et al*. Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. *Circ Res* 2002;**91**:434-440.

Taddei S, Virdis A, Ghiadoni L, Magagna A, Salvetti A. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation* 1998;**97**:2222-2229.

Tatemoto K, Hosoya M, Habata Y *et al*. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem Biophys Res Commun* 1998;**251**:471-476.

Tatemoto K, Takayama K, Zou MX *et al*. The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regul Pept* 2001;**99**:87-92.

Thomas SHL. Impedance cardiography using the Sramek-Bernstein method: accuracy and reproducibility at rest and during exercise. *Br J Clin Pharmacol* 1992; **34**:467-476.

Ueda S, Wada A, Umemura S. Methodological validity and feasibility of the nitric oxide clamp technique for nitric oxide research in human resistant vessels. *Hypertens Res* 2004;**27**:351-357.

Vallance P, Collier J, Moncada S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* 1989;**2**:997-1000.

Van Coillie E, Proost P, Van Aelst I *et al*. Functional comparison of two human monocyte chemotactic protein-2 isoforms, role of the amino-terminal pyroglutamic acid and processing by CD26/dipeptidyl peptidase IV. *Biochemistry* 1998;**37**: 12672-12680.

Vickers C, Hales P, Kaushik V *et al*. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* 2002;**277**:14838-14843.

Walker HA, Jackson G, Ritter JM, Chowienczyk PJ. Assessment of forearm vasodilator responses to acetylcholine and albuterol by strain gauge plethysmography: reproducibility and influence of strain gauge placement. *Br J Clin Pharmacol* 2001;**51**:225-229.

Wang G, Qi X, Wei W, Englander EW, Greeley GH, Jr. Characterization of the 5'-regulatory regions of the rat and human apelin genes and regulation of breast apelin by USF. *FASEB J* 2006;**20**:2639-2641.

Wei L, Hou X, Tatemoto K. Regulation of apelin mRNA expression by insulin and glucocorticoids in mouse 3T3-L1 adipocytes. *Regul Pept* 2005;**132**:27-32.

Wilkinson IB, Webb DJ. Venous occlusion plethysmography in cardiovascular research: methodology and clinical applications. *Br J Clin Pharmacol* 2001;**52**: 631-646.

Wittstein IS, Kass DA, Pak PH *et al*. Cardiac nitric oxide production due to angiotensin-converting enzyme inhibition decreases beta-adrenergic myocardial contractility in patients with dilated cardiomyopathy. *J Am Coll Cardiol* 2001;**38**:429-435.

Woltjer HH, Bogaard HJ, de Vries PM. The technique of impedance cardiography. *Eur Heart J* 1997;**18**:1396-1403.

Xie H, Tang SY, Cui RR *et al*. Apelin and its receptor are expressed in human osteoblasts. *Regul Pept* 2006;**134**:118-125.

Xie H, Yuan LQ, Luo XH *et al*. Apelin suppresses apoptosis of human osteoblasts. *Apoptosis* 2007;**12**:247-254.

Young JB, Dunlap ME, Pfeffer MA *et al*. Mortality and morbidity reduction with Candesartan in patients with chronic heart failure and left ventricular systolic dysfunction: results of the CHARM low-left ventricular ejection fraction trials. *Circulation* 2004;**110**:2618-2626.

Yue P, Jin H, Aillaud M *et al*. Apelin is necessary for the maintenance of insulin sensitivity. *Am J Physiol Endocrinol Metab* 2010;**298**:E59-67.

Yue P, Jin H, Xu S *et al*. Apelin decreases lipolysis via G(q), G(i), and AMPK-Dependent Mechanisms. *Endocrinology* 2011;**152**:59-68.

Zeng XX, Wilm TP, Sepich DS, Solnica-Krezel L. Apelin and its receptor control heart field formation during zebrafish gastrulation. *Dev Cell* 2007;**12**:391-402.

Zhong JC, Huang Y, Yung LM *et al*. The novel peptide apelin regulates intrarenal artery tone in diabetic mice. *Regul Pept* 2007;**144**:109-114.

Zhou N, Fan X, Mukhtar M *et al*. Cell-cell fusion and internalization of the CNS-based, HIV-1 co-receptor, APJ. *Virology* 2003;**307**:22-36.

PUBLICATIONS

Japp AG, Cruden NL, Barnes G *et al.* Acute cardiovascular effects of apelin in humans: potential role in patients with chronic heart failure. *Circulation* 2010;**121**:1818-1827.

Barnes G, **Japp AG,** Newby DE. Translational promise of the apelin-APJ system**.** *Heart* 2010;**96**:1011-1016.

Japp AG, Cruden NL, Amer DA *et al*. Vascular effects of apelin in vivo in man. *J Am Coll Cardiol* 2008;**52**:908-913.

Japp AG, Newby DE. The apelin-APJ system in heart failure: pathophysiologic relevance and therapeutic potential. *Biochem Pharmaco*. 2008;**75**:1882-1892.